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Award Number: DAMD17-98-1-8206

TITLE: The Regulation and Function of Nuclear Receptor  
Corepressor SMRT in Human Breast Cancer Cells

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REPORT DATE: May 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

**1. AGENCY USE ONLY (Leave blank)****2. REPORT DATE**

May 2000

**3. REPORT TYPE AND DATES COVERED**

Annual Summary (1 May 99 - 30 Apr 00)

**4. TITLE AND SUBTITLE**

The Regulation and Function of Nuclear Receptor Corepressor SMRT in Human Breast Cancer Cells

**5. FUNDING NUMBERS**

DAMD17-98-1-8206

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**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING  
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES**

This report contains colored photographs in the appendices

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

Steroid and nuclear receptor coactivators have been implicated in the regulation of nuclear receptor function by enhancing ligand-dependent transcriptional activation of target gene expression. We have previously isolated receptor-associated coactivator 3 (RAC3), which belongs to the steroid receptor (SRC) family. In the current study, we have investigated the differential mechanisms by which RAC3 interacts with and modulates the transcriptional activity of different nuclear receptors. We found that the vitamin D receptor (VDR) and estrogen receptor beta (Erb) interact with different alpha-helical LXXLL motifs of RAC3. Peptides corresponding to these motifs have diverse affinities for the VDR and Erb and mutation of specific motifs differentially impairs the ability of RAC3 to interact with these receptors in vitro. Consequently, these mutations inhibit the enhancement of transcriptional activation by receptors in vivo. Furthermore, we found enhancement of transcriptional activation by receptors in vivo. Furthermore, we found that the activation function-2 (AF-2) domain of the retinoid X receptor (RXR) interferes with RAC3 binding to a DNA-bound VDR/RXR heterodimer, while the VDR AF-2 domain is required for this interaction. These results suggest a receptor-specific binding preference for the different LXXLL motifs of RAC3, which may provide flexibility for RAC3 to differentially regulate the function of different nuclear receptors.

**14. SUBJECT TERMS**

Breast Cancer

**15. NUMBER OF PAGES**

44

**16. PRICE CODE**

Key words: coactivator, RAC3, LXXLL motif, VDR, ER-beta

**17. SECURITY CLASSIFICATION  
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION  
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION  
OF ABSTRACT**

Unclassified

**20. LIMITATION OF ABSTRACT**

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

20010301 112

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## Introduction

Members of the steroid receptor coactivator (SRC) family have been implicated in the regulation of nuclear receptor function by enhancing ligand-dependent transcriptional activation of target gene expression. We have previously isolated the third member of the SRC family, termed receptor-associated coactivator 3 (RAC3), which interacts with both receptors and the general coactivator CBP via distinct domains. In this study, we investigated the differential mechanisms by which RAC3 may interact with and modulate the transcriptional activity of different nuclear receptors. We found that the vitamin D receptor (VDR) and estrogen receptor beta (ER $\beta$ ) interact with different  $\alpha$ -helical LXXLL motifs of RAC3. Peptides corresponding to these motifs have diverse affinities for the VDR and ER $\beta$  and mutation of specific motifs differentially impairs the ability of RAC3 to interact with these receptors in vitro. Consequently, these mutations also inhibit the enhancement of transcriptional activation by these receptors in vivo. In addition, we found that the activation function-2 (AF-2) domain of the retinoid X receptor (RXR) interferes with RAC3 binding to a DNA-bound VDR/RXR heterodimer, while the VDR AF-2 domain is required for this interaction. These results suggest a receptor-specific binding preference for the different LXXLL motifs of the RAC3 receptor-interacting domain and that RAC3 may differentially regulate the function of nuclear receptors via multiple LXXLL motifs.

## Annual Summary

Coactivators recruited by ligand-bound nuclear receptors include members of the SRC family of coactivators such as SRC1 (also known as NCoA-1), TIF2/GRIP1 (also known as SRC2 or NCoA-2), and RAC3/ACTR/pCIP/AIB1 (also known as SRC3 or NCoA-3). Intriguingly, these coactivators have been found to contain several conserved motifs, termed NR boxes, with the sequence LXXLL, where X is any amino acid. Motifs within the receptor interacting domain and transcriptional activation domains of SRC1 and TIF2 have been demonstrated to mediate interactions with liganded nuclear receptors and CBP/p300, respectively. Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic  $\alpha$ -helices with the leucine residues comprising a hydrophobic surface on one face of the helix. The helical motif is able to interact with the AF-2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding. Mutational analyses of the NR boxes of SRC1 and TIF2/GRIP1 have also uncovered a receptor-specific code of interaction, where different nuclear receptors require different NR boxes to interact with coactivator. In this study, we investigate the mechanisms by which RAC3 regulates the function of the VDR and ER $\beta$ , for little is known concerning the regulation of these receptors by SRC coactivators, particularly RAC3. These analyses also reveal receptor-specific interactions in which the VDR and ER $\beta$  interact with different surfaces of RAC3. We demonstrate different preferences of these receptors for specific NR boxes of RAC3 and that single mutations in these LXXLL motifs are able to severely impair the ability of RAC3 to interact with and thus, coactivate, the VDR and ER $\beta$ . In analyzing the requirement of nuclear receptor AF-2 domains, we observe that the AF-2 domain of RXR can inhibit RAC3-RID binding to the DNA-bound VDR/RXR heterodimer, while the AF-2 domain of VDR is absolutely required for this interaction. These data add complexity to the regulation of nuclear receptor activity by SRC coactivators and suggest that different classes of nuclear receptors may be differentially regulated by RAC3.

### VDR and ER $\beta$ interact with multiple surfaces of RAC3

We have previously defined the minimal receptor interacting domain (RID) of RAC3 to be amino acids 613-752, which contains the first three LXXLL motifs. We wished to further determine if different receptors were capable of binding to the same regions of RAC3. To accomplish this, we purified a panel of GST-RAC3 fusion proteins, in total comprising the full-length RAC3, and probed these fusions with  $^{35}\text{S}$ -methionine labeled VDR and ER $\beta$  in a Far-Western assay. The VDR, as expected, interacted in a ligand-dependent manner with GST-RAC3 613-752 in this assay. It also bound GST-RAC3 723-1034, which only contains NR box iii, in a

ligand-dependent manner. The VDR did not interact with any other GST-RAC3 fragment, including GST-RAC3 342-646, which contains NR box i. It also appeared that the VDR interacted more strongly with GST-RAC3 723-1034 than with GST-RAC3 613-752, suggesting a more important role for NR box iii in the RAC3-VDR interaction. However, a different pattern was evident upon repeating this assay with  $^{35}\text{S}$ -ER $\beta$ , for in addition to ligand-dependent interactions with GST-RAC3 613-752 and 723-1034, ER $\beta$  also bound the 342-646 fragment, which contains only NR box i. These interactions were of approximately equal intensity. There was also a weak, ligand-independent interaction with GST-RAC3 1-407. Identical results were obtained for ER $\alpha$ . A Coomassie blue-stained polyacrylamide gel of the GST-RAC3 fusion protein confirmed the identity of each GST-RAC3 fusion protein and approximately equal protein concentrations in each lane. Thus, the VDR and ER $\beta$  display different binding patterns for RAC3 fragments, with the VDR interacting preferentially with regions containing NR box iii and the ER $\beta$  interacting with regions containing all three NR boxes and the N-terminal bHLH-PAS domain.

### **NR Box peptides differentially compete with nuclear receptors for RAC3 binding**

We then wanted to investigate the relative importance of individual NR boxes within the RAC3-RID in mediating the interactions between RAC3 and the VDR or ER $\beta$ . Peptides were synthesized corresponding to NR boxes i, ii, and iii, which were incubated with  $^{35}\text{S}$ -labeled nuclear receptor and 1 $\mu\text{M}$  ligand prior to probing the GST-RAC3 613-752 fragment in the Far-Western assay. With the VDR, peptides corresponding to the second and third LXXLL motif were able to compete away the VDR-RAC3 RID interaction in a dose-dependent manner. Upon quantifying the data, it was evident that peptide iii was a more potent inhibitor than peptide ii, while the peptide comprising NR box i had little, if any, effect on the VDR-RID interaction. A control experiment demonstrated that the effects of these peptides were specific, for a random peptide did not alter the interaction between the  $^{35}\text{S}$ -VDR and GST-RAC3-RID. We again identified a different pattern when the same experiment was done with the ER $\beta$ . Here, all three peptides were able to compete efficiently for ER $\beta$  binding with the RAC3-RID, with peptide ii being the most potent. Thus, these data reveal receptor-specific preferences for interactions between RAC3 and different nuclear receptors, for the VDR and ER $\beta$  have different affinities for the NR boxes of RAC3.

### **NR box mutations can impair RAC3 interactions with nuclear receptors in vitro**

In order to assess the integrity of the LXXLL motif in mediating the interaction between the RAC3-RID and nuclear receptors, we used site-directed mutagenesis to switch the leucine residues of each motif to

alanines. The mutants were made using the GST-RAC3-RID fusion as the template and tested for their ability to interact with the VDR or ER $\beta$  in GST-pulldown assays. The wild-type RAC3-RID was able to pull down a significant amount of  $^{35}\text{S}$ -VDR in the presence of 1 $\mu\text{M}$  Vitamin D. This interaction was specific, for GST alone pulled down much less  $^{35}\text{S}$ -VDR. Mutations in NR boxes i or ii displayed wild-type binding. However, when NR box iii was mutated, the RID-VDR interaction was greatly reduced to a level only slightly higher than background binding to GST alone. GST-RAC3 342-646, which contains only NR box i, also had minimal binding to the VDR, consistent with the Far-Western assay. Equal protein concentrations of each GST fusion confirmed the specificity of these findings. Thus, these data support the above observations in implicating NR box iii as being most critical to RAC3 interaction with the VDR.

The wild-type RID was also able to pull down significant amounts of  $^{35}\text{S}$ -ER $\beta$  in the GST-pulldown assay. In contrast to the VDR, alanine substitution for leucine in any of the three NR boxes weakened the interaction of the RAC3-RID with ER $\beta$ , with the mutation of NR box ii being the most deleterious, again supporting the results of LXXLL peptide competition experiments. However, with each mutation, significant binding above background between the RAC3-RID and  $^{35}\text{S}$ -ER $\beta$  was still observed. Furthermore, the GST-RAC3 342-646 fragment, with only NR box i, was able to interact efficiently with ER $\beta$  as in the Far-Western assay. These data suggest that although all three motifs are capable of interacting with ER $\beta$ , none them are absolutely required for the interaction. In contrast, NR box iii of RAC3 appears to be essential for the interaction with the VDR.

### **RAC3-RID interactions with DNA-bound nuclear receptors**

The above data provide compelling evidence that the VDR interacts specifically with RAC3 in solution via the LXXLL motifs of the RAC3 RID, particularly NR box iii. To gain further insight into the function of NR boxes in coactivator-VDR interactions on a heterodimeric complex bound to DNA, we performed gel-shift assays with VDR/RXR heterodimers on a DR3 element in the presence of wild-type or mutant RAC3-RID. Addition of the RAC3-RID resulted in a ligand-dependent shift of the heterodimeric complex to a slower migrating form. Mutation of NR box i had little effect on the ability of the RID to shift the complex. However, mutating NR box ii diminished somewhat the formation of the RID-VDR/RXR complex, while mutating NR box iii nearly abolished formation completely. This result differs from the GST-pulldown, in which only the NR box iii mutation inhibited interaction with VDR alone, suggesting that motif ii may contribute to the interaction with DNA-bound VDR/RXR.



We next analyzed the involvement of nuclear receptor AF-2 domains in regulating the interaction between the RAC3-RID and the VDR/RXR heterodimer. Using the gel-shift assay, we compared the ability of the RID to bind the wild-type heterodimer versus the VDR/RXR443 and VDR402/RXR heterodimers, in which the AF-2 domain of RXR or VDR had been deleted, respectively. Deletion of the VDR AF-2 domain resulted in the loss of the RID-shifted complex, suggesting that the VDR AF-2 domain is required for interaction of RAC3 with the heterodimer, and that RXR AF-2 domain alone is not sufficient for the interaction. Interestingly, deletion of the RXR AF-2 domain resulted in a much stronger shift of the heterodimeric complex by the RAC3-RID, suggesting that the RXR AF-2 domain can inhibit the interaction between RAC3 and VDR/RXR.

Finally, we compared the RAC3 NR box preferences of VDR/RXR versus VDR/RXR443. With wild-type receptors, the same pattern was observed as above. The NR box i mutation had little effect on the ability of RAC3-RID to shift the VDR/RXR complex. Conversely, mutation of NR box ii impaired the interaction, while mutation of NR box iii nearly abolished it completely. Intriguingly, the VDR/RXR443 heterodimer displayed different NR box preferences. Mutation of NR box i or iii greatly reduced the shift by the RAC3-RID, while mutation of NR box ii only slightly weakened the binding. Thus deletion of the RXR AF-2 domain resulted in a switch in the NR box requirements, with NR boxes i and iii being most important for VDR/RXR443 compared to NR boxes ii and iii for VDR/RXR. This finding supports the hypothesis that multiple LXXLL motifs provide RAC3 with the flexibility to adapt to different receptor dimers.

### **Effects of NR box mutations on RAC3 coactivation function in vivo**

RAC3 has previously been shown to enhance the transcriptional activity of the retinoic acid receptor (RAR) and progesterone receptor (PR). However, its effect on VDR and ER $\beta$  function in vivo has not been demonstrated. To address this, we performed transient transfection assays in HEK293 and CV-1 cells using luciferase reporters harboring either two copies of the VDRE of the osteopontin gene for VDR studies or a consensus ERE element for ER $\beta$  studies. Transfection of the VDR into HEK293 cells minimally activated the VDRE-driven reporter. However, treating these cells with Vitamin D strongly stimulated its activity. Cotransfection of RAC3 further enhanced VDR transcriptional activation 3-fold, consistent with the coactivation capabilities of the other SRC family members. In CV-1 cells, coactivation by RAC3 was not as potent, but RAC3 still enhanced VDR activity by approximately 50%. Therefore, RAC3 can function as a coactivator of the VDR in multiple types of mammalian cells.

We then analyzed the role of the NR boxes in mediating the ability of RAC3 to potentiate VDR activity. Mutations of each NR box of the RAC3-RID were made in the context of the full-length RAC3 protein and tested for their ability to coactivate the VDR in transient transfection assays. Mutation of NR box i did not inhibit RAC3 enhancement of VDR transactivation, consistent with its inability to block the interaction of RAC3 with VDR in vitro. However, the RAC3- NR box ii or iii mutations greatly reduced the function of RAC3 in enhancing VDR activity. Although the mutation of NR box ii did not affect the VDR-RID interaction in GST-pulldown assays, it did reduce binding to the VDR/RXR heterodimer in the more functionally relevant gel-shift assay. Also, a peptide corresponding to NR box ii was able to efficiently compete with the RAC3-RID for VDR binding. Taken together with the above in vivo data, it is clear that NR boxes ii and iii are both involved in RAC3 regulation of the VDR, while the role of NR box i appears minimal.

We then conducted these experiments with the ER $\beta$  in CV-1 cells. Estradiol treatment of CV-1 cells transfected with the ER $\beta$  and the ERE-Luciferase reporter activated reporter expression approximately 8-fold. Cotransfection of wild-type RAC3 resulted in a strong enhancement of ER $\beta$  activity. It is evident from this data that RAC3 is a more potent coactivator for the ER $\beta$  than for the VDR. Cotransfection of RAC3 expression plasmids containing mutations in NR boxes i, ii, or iii all suppressed the ability of RAC3 to coactivate the ER $\beta$ , with the NR box ii mutant having the greatest and NR box iii mutant having more modest effects on RAC3 function. However, all three mutant coactivators were still able to somewhat enhance ER $\beta$  activity. Thus, this in vivo data correlates with the in vitro data in implicating all three NR boxes of the RAC3 receptor-interacting domain as being important, but not required, for RAC3 regulation of ER $\beta$ .

### **Key Research Accomplishments**

- Analyzed the mechanism of receptor-associated coactivator 3 (RAC3) interaction with the vitamin D receptor (VDR) and estrogen receptor beta (ER $\beta$ )
- Identified different binding surfaces of RAC3 for the VDR and ER $\beta$
- Established the LXXLL motifs of RAC3 as being critical to interactions with nuclear receptors
- Demonstrated that peptides corresponding to LXXLL motifs ii and iii can compete for VDR interaction with RAC3, with peptide iii being much more potent, while peptides corresponding to LXXLL motifs i, ii, and iii can compete for ER $\beta$  interaction
- Demonstrated that mutation of RAC3 LXXLL motif iii nearly abolishes VDR interaction with RAC3 in vitro while mutation of any of the three LXXLL motifs reduces, but does not abolish, the interaction with ER $\beta$
- Demonstrated that mutation of RAC3 LXXLL motif ii or iii inhibits RAC3 interaction with a DNA-bound VDR/RXR heterodimer
- Demonstrated that helix 12 of VDR is required, while helix 12 of RXR inhibits, interaction of RAC3 with a DNA-bound VDR/RXR heterodimer
- Demonstrated that mutation of RAC3 LXXLL motifs ii or iii abolishes the ability of RAC3 to enhance transcription by the VDR in vivo, while mutation of any of the three LXXLL motifs reduces, but does not abolish, RAC3 coactivation of ER $\beta$  activity

### **Reportable Outcomes**

1. Leo, C. and Chen, J.D. (2000) The SRC family of nuclear receptor coactivators. *Gene* 245, 1-11.
2. Leo, C., Li, H., and Chen, J.D. (2000) Differential Mechanisms of Nuclear Receptor Regulation by Receptor-associated Coactivator 3. *The Journal of Biological Chemistry* 275, 5976-5982.
3. Li, H.\*, Leo, C.\*, Zhu, J., Wu, X., O'Neil, J., Park, E., and Chen, J.D. (2000) Sequestration and Inhibition of Daxx-mediated Transcriptional Repression by PML. *Molecular and Cellular Biology* 20, 1784-1796. (\*Co-first authors)

## Appendices

Review

# The SRC family of nuclear receptor coactivators

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Received 2 November 1999; received in revised form 3 January 2000; accepted 13 January 2000

Received by A.J. van Wijnen

## Abstract

Nuclear hormone receptors are ligand-dependent transcription factors that regulate genes critical to such biological processes as development, reproduction, and homeostasis. Interestingly, these receptors can function as molecular switches, alternating between states of transcriptional repression and activation, depending on the absence or presence of cognate hormone, respectively. In the absence of hormone, several nuclear receptors actively repress transcription of target genes via interactions with the nuclear receptor corepressors SMRT and NCoR. Upon binding of hormone, these corepressors dissociate away from the DNA-bound receptor, which subsequently recruits a nuclear receptor coactivator (NCoA) complex. Prominent among these coactivators is the SRC (steroid receptor coactivator) family, which consists of SRC-1, TIF2/GRIP1, and RAC3/ACTR/pCIP/AIB-1. These cofactors interact with nuclear receptors in a ligand-dependent manner and enhance transcriptional activation by the receptor via histone acetylation/methylation and recruitment of additional cofactors such as CBP/p300. This review focuses on the mechanism of action of SRC coactivators in terms of interactions with receptors and activation of transcription. Specifically, the roles of the highly conserved LXXLL motifs in mediating SRC function will be detailed. Additionally, potential diversity among SRC family members, as well as several recently cloned SRC-associated cofactors, will be discussed. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** CBP; LXXLL motif; RAC3/ACTR/pCIP/AIB-1; Steroid receptor coactivator; TIF2/GRIP1

## 1. Introduction

The steroid and thyroid nuclear receptor superfamily is a large class of ligand-dependent transcription factors involved in the regulation of genes that play critical roles in a wide array of biological processes, including development, reproduction, and homeostasis (Mangelsdorf et al., 1995). This superfamily can be

further subdivided into three classes of nuclear receptors. Type I or steroid receptors include those for estrogens (ER), progestins (PR), androgens (AR), glucocorticoids (GR), and mineralcorticoids (MR). Type I receptors are coupled to heat-shock proteins and sequestered to the cytoplasm in the absence of ligand (Tsai and O'Malley, 1994). Upon hormone binding, they dissociate from the heat-shock proteins, homodimerize, and translocate to the nucleus, where they bind cognate response elements consisting of palindromic repeats. Type II receptors include those for all-*trans* retinoic acid (RAR), thyroid hormone (TR), and vitamin D (VDR). These receptors are strictly nuclear and form heterodimers with the receptor for 9-*cis* retinoic acid (RXR). They also bind constitutively to response elements consisting of direct repeats. A third class of nuclear receptors is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

Most members of the nuclear receptor superfamily share a common domain structure. The N-terminus

Abbreviations: ACTR, activator of retinoic acid receptor; AF-2, activation function-2 domain; AIB-1, amplified in breast cancer-1; CARM1, coactivator-associated arginine methyltransferase; GRIP1, glucocorticoid receptor-interacting protein 1; HAT, histone acetyltransferase; LBD, ligand binding domain; NR box, nuclear receptor interacting box; pCIP, p300/CBP cointegrator-associated protein; RAC3, receptor associated coactivator 3; SRA, steroid receptor RNA activator; SRC, steroid receptor coactivator; TIF2, transcription intermediary factor 2; TRAM-1, thyroid receptor activator molecule 1.

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contains the variable A/B region, which also includes the ligand-independent activation function-1 (AF-1) domain. The C region represents the highly conserved DNA-binding domain (DBD) and is followed by the hinge region (D) and the C-terminal ligand-binding domain (LBD) (E). The LBD contains the ligand-dependent activation function-2 (AF-2) domain and also mediates dimerization of nuclear receptors. In the absence of ligand, several nuclear receptors are able to repress basal transcription via recruitment of the nuclear receptor corepressors SMRT and NCoR (Horlein et al., 1995; Chen and Evans, 1995). SMRT and NCoR are found in complexes with the corepressor mSin3 and histone deacetylases (HDACs), suggesting that transcriptional repression by nuclear receptors may involve histone deacetylation (Nagy et al., 1997; Heinzel et al., 1997; Alland et al., 1997). Ligand binding triggers the release of these corepressors and subsequent recruitment of coactivators through a drastic conformational change in the AF-2 domain of the receptor. Structural studies have demonstrated that helix 12 within the AF-2 domain projects away from the LBD in the unliganded structure (Renaud et al., 1995; Wagner et al., 1995; Bourguet et al., 1995; Brzozowski et al., 1997). This helix rotates nearly 180° to pack tightly against the LBD upon hormone binding in the RAR, TR, and ER crystal structures. This conformational change, together with ligand-induced changes in helices 3–5, is believed to facilitate interactions of the receptor with coactivators (Shiau et al., 1998; Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998). Subsequently, coactivators are able to enhance transcriptional activation by the receptor via mechanisms that include recruitment of the general coactivator CBP/p300 and histone acetylation.

Coactivators are generally defined as proteins that can interact with DNA-bound nuclear receptors and enhance their transcriptional activation function. Although many nuclear receptor coactivators have been identified (McKenna et al., 1999), the steroid receptor coactivator (SRC) family has been the focus of intense study in recent years. Thus this review will focus on the mechanisms of action of these cofactors in regulating the function of nuclear receptors and also highlight several of the recently cloned SRC-associated proteins.

## 2. The SRC family of coactivators

The first nuclear receptor coactivator, steroid receptor coactivator-1 (SRC-1), was cloned by using the PR-LBD as bait in a yeast-two-hybrid screen of a human B-cell cDNA library (Oñate et al., 1995). SRC-1 interacts in a ligand-dependent manner with and enhances AF-2 transcriptional activation by a broad range of nuclear receptors, including PR, ER, TR, RXR, GR, and PPAR. Recent data also detail the enhancement of ER (Webb

et al., 1998) and AR (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999) AF-1 activities by SRC-1. In addition, SRC-1 has been demonstrated to interact with the general transcription factors TBP and TFIIB, although the functional consequences of these interactions are unknown (Takeshita et al., 1996; Ikeda et al., 1999). Furthermore, SRC-1 is able to enhance transcriptional activation mediated by NF- $\kappa$ B, SMAD3, and AP-1 (Lee et al., 1998; Yanagisawa et al., 1999; Na et al., 1998), supporting a role for nuclear receptor coactivators in multiple intracellular signaling pathways. Subsequent studies have identified two functionally distinct SRC-1 isoforms, SRC-1a and SRC-1c, which contain unique C-termini, suggesting that alternative splicing may regulate SRC-1 function (Kalkhoven et al., 1998).

The identification of transcription intermediary factor 2 (TIF2) and GR-interacting protein 1 (GRIP1) established the SRC family of coactivators (Voegel et al., 1996; Hong et al., 1996). TIF2 was isolated in a Far-western screen as an ER- and RAR-interacting factor, while GRIP1 was isolated using the GR-LBD as bait in a yeast-two-hybrid screen. TIF2 and GRIP1 share 94% amino acid identity, thus represent the human and murine orthologs, respectively. TIF2 and GRIP1 associate in vivo with hormone-bound RAR, ER, and PR and coactivate ligand-dependent transactivation. Like SRC-1, GRIP1 also has been demonstrated to enhance receptor AF-1 activity in addition to that of the AF-2 domain (Ma et al., 1999). Intriguingly, the inv(8)(p11q13) chromosomal translocation results in a fusion between TIF2 and the MOZ gene, which contributes to the pathogenesis of acute myeloid leukemia (AML), suggesting a role for transcriptional regulation by nuclear receptor coactivators in these leukemias (Carapeti et al., 1998).

The third member of the SRC family was reported simultaneously by several groups as an RAR-interacting protein (RAC3), a CBP-interacting protein (p/CIP), a hRAR $\beta$ -stimulatory protein (ACTR), a gene amplified in breast cancer (AIB-1), and a TR-interacting protein (TRAM-1) (Li et al., 1997; Torchia et al., 1997; Chen et al., 1997; Anzick et al., 1997; Takeshita et al., 1997). p/CIP represents the mouse homolog, while RAC3/ACTR/AIB-1/TRAM are human isoforms. In addition to coactivating many nuclear receptors, pCIP has also been demonstrated to enhance the activity of interferon- $\alpha$  and cAMP regulatory element binding protein (CREB), suggesting that this coactivator may be involved in multiple signaling pathways (Torchia et al., 1997). Furthermore, RAC3/TRAM-1 expression can be upregulated by hormone treatment, which represents another possible mechanism by which coactivators may potentiate hormone action (Li and Chen, 1998; Misiti et al., 1998).

### 3. The LXXLL motif

The SRC family of coactivators also shares a common domain structure, with the most highly conserved region being the N-terminal bHLH–PAS domain (Fig. 1a). The bHLH region functions as a DNA-binding or dimerization surface in many transcription factors, including the MyoD family of proteins (Murre et al., 1989a, b). The PAS motif is also found in several transcriptional regulators, including Period (Per), Aryl hydrocarbon receptor (AhR), and single-minded (Sim). Similar to the bHLH domain, the PAS domain also plays a role in protein–protein interactions and dimerization. However, the function of the bHLH–PAS domains of SRC coactivators remains unknown, though it is likely to mediate intra- or intermolecular interactions. This bHLH–PAS

domain is followed by a centrally located receptor-interacting domain (RID) and C-terminal transcriptional activation domain (AD), which will be discussed in detail below.

The RID of SRC coactivators mediates ligand-dependent, direct interactions with nuclear receptors (Li and Chen, 1998; Voegel et al., 1998; Oñate et al., 1998). Intriguingly, detailed analysis of the sequence of the RID identified a conserved motif, LXXLL, where L is leucine and X is any amino acid, that is termed the NR box (Heery et al., 1997) (Fig. 1b). Three such motifs are found in the RID of SRC coactivators, with an additional, non-conserved NR box also present at the C-terminus of the SRC-1 isoform SRC-1a. Site-directed mutagenesis and peptide competition experiments have provided strong evidence for the requirement of these

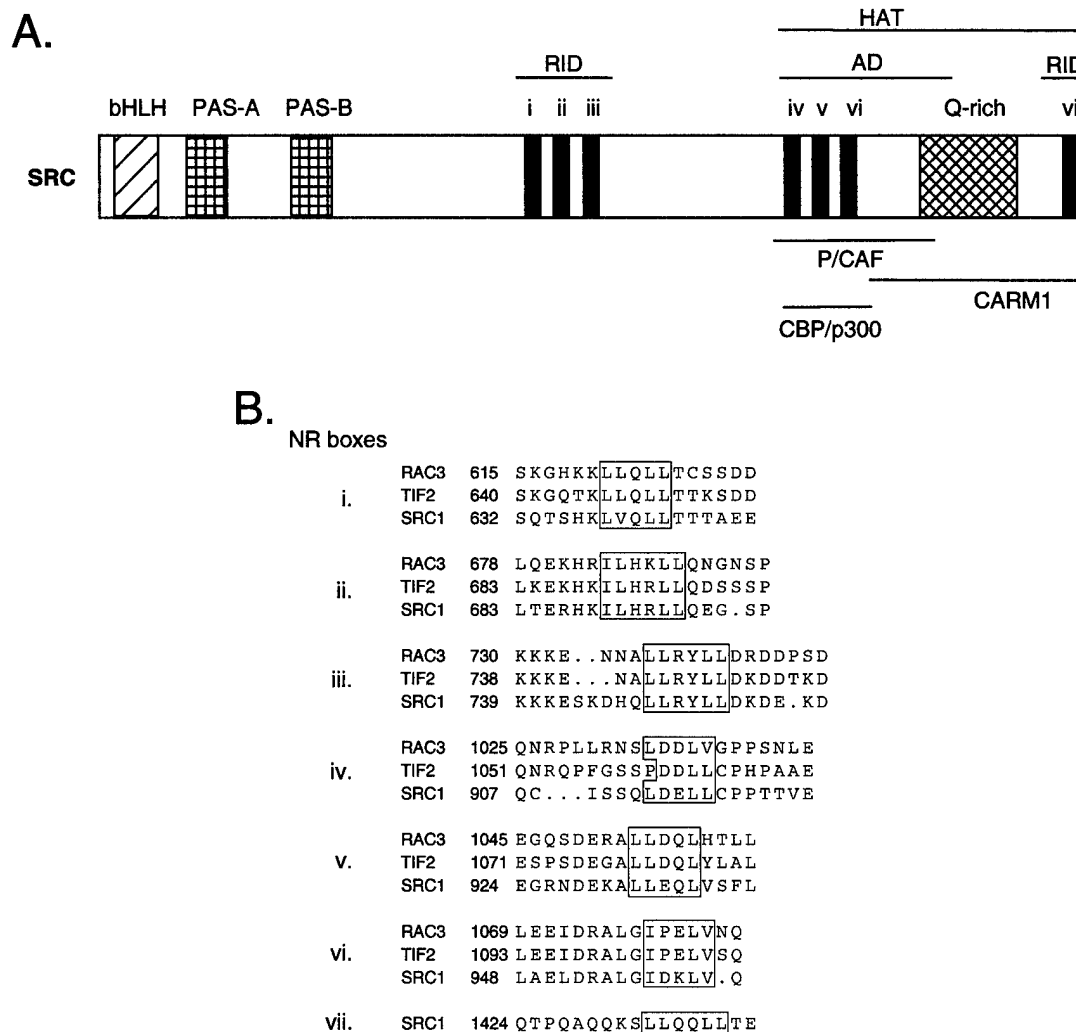


Fig. 1. SRC family domain structure. (A) Schematic representation of the structural domains of SRC coactivators. The N-terminus contains the highly conserved bHLH and PAS A/B domains. The centrally located receptor-interacting (RID) and activation (AD) domains each contain three LXXLL motifs, while SRC-1 contains an additional, non-conserved motif at the C-terminus. The C-terminus contains a glutamine-rich domain. The specific domains for interaction with P/CAF, CBP/p300, and CARM1, as well as the histone acetyltransferase (HAT) domain, are indicated. (B) Sequence alignment of the SRC LXXLL motifs. The starting amino acids are in parentheses. Motifs i–iii are located in the receptor-interacting domain and motifs iv–vi are found in the transcriptional activation domain. SRC-1 contains an additional non-conserved motif at its C-terminus.



motifs for mediating interactions between coactivators and liganded nuclear receptors (Heery et al., 1997; Torchia et al., 1997; Ding et al., 1998). Further support for the role of these motifs in mediating agonist-dependent interactions with nuclear receptors is found in a study in which phage-displayed peptide libraries were screened for peptides that interact specifically with agonist or antagonist bound estrogen receptor (Norris et al., 1999). Many peptides isolated with estradiol-bound ER $\alpha$  contained the LXXLL motif, while those isolated with tamoxifen-bound receptor did not. These findings suggest that the activation of the ER $\alpha$  by tamoxifen that is observed in some tissues might occur via a different mechanism than estradiol-induced activation, such as through the recruitment of non-LXXLL containing coactivators to tamoxifen-specific surfaces of the ER. Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic  $\alpha$ -helices with the leucine residues comprising a hydrophobic surface on one face of the helix. The helix is able to interact with the AF-2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding (Torchia et al., 1997; Shiau et al., 1998; Nolte et al., 1998; Darimont et al., 1998; Feng et al., 1998).

The most interesting aspect of NR box function is the revelation that a receptor-specific code exists, where different nuclear receptors prefer different NR boxes of the RID for interaction with coactivators (Leers et al., 1998; Ding et al., 1998; Darimont et al., 1998; McNerney et al., 1998). For example, a 13-aa peptide encompassing GRIP1 motif ii efficiently blocked interaction between GRIP1 and the TR $\beta$ -LBD in vitro, while a peptide comprising motif iii was a more potent competitor for GR binding (Darimont et al., 1998). Similarly, yeast-two-hybrid assays demonstrate that mutation of TIF2 motif ii is most deleterious to interactions with PPAR $\alpha$ , while a motif i mutation has the greatest effect on the TIF2-RXR $\beta$  interaction (Leers et al., 1998). In all cases, however, mutation of a single motif does not completely abolish coactivator interaction with nuclear receptors, suggesting that multiple NR boxes contribute to the overall, high-affinity binding to the receptor. It is likely that the precise arrangement of multiple motifs and structural nuances of each receptor determines the relative contribution of each NR box to the interaction.

This receptor-specific code has also been analyzed in vivo in terms of transcriptional coactivation of nuclear receptors by SRC-1 via site-directed mutagenesis and antibody microinjection assays (McNerney et al., 1998). The requirement of specific NR boxes for transactivation of reporter genes by different receptors was determined by injecting anti-SRC-1 antibodies into cells along with rescuing plasmids for wild-type or NR box mutants of SRC-1. Anti-SRC-1 IgG completely abolishes transcrip-

tional activation by ER, PR, RAR, TR, and PPAR $\gamma$ , while coinjection of wild-type SRC-1 rescues receptor function. Mutation of NR box ii prevented rescue of ER function in SRC-1 immunodepleted cells, while NR boxes ii and iii were required for rescue of RAR and TR activity and boxes i and ii for PR activity. Furthermore, in the case of PPAR $\gamma$ , different ligands elicited different NR box requirements for SRC-1 coactivation. Troglitazone-bound PPAR $\gamma$  preferred NR box ii over box i, while the opposite was observed in indomethacin-treated cells. Together, these data support receptor-specific LXXLL motif requirements for coactivation function and receptor interactions that account for the presence of multiple NR boxes within SRC coactivators and imply that these motifs do not serve merely redundant functions.

Other determinants that contribute to the specificity of NR box selectivity by different nuclear receptors include residues flanking each NR box. For instance, a chimeric peptide containing the GRIP1 NR box iii motif in the context of the flanking sequences of NR box ii competed for TR-LBD binding with a similar potency as the peptide comprising NR box ii (Darimont et al., 1998). Also, using phage-displayed libraries enriched for LXXLL-containing peptides, it was demonstrated that several subclasses of these peptides exist which contain different flanking residues and which vary in their abilities to interact with different ER mutants and other receptors (Chang et al., 1999). Furthermore, it has been shown that the flanking N-terminal amino acids are not essential, while the eight residues C-terminal to the NR box are required for SRC-1 mediated coactivation of RAR, TR, and ER (McNerney et al., 1998). These studies also revealed additional preferences of ER and RAR for different NR box ii C-terminal amino acids. Intact residues +12 and +13 (where L of LXXLL is +1) are required for SRC-1 rescue of ER activity, while residues at +6, +7, +11, and +13 are necessary for rescue of RAR function. Finally, since most nuclear receptors require two intact NR boxes of coactivator for interaction, the spacing between the motifs also can serve as a determinant for recognition. Deletion of 30 of the 50 amino acids between NR boxes ii and iii abolished the ability of SRC-1 to rescue IgG-mediated inhibition of RAR activity (McNerney et al., 1998). In contrast, proper spacing between NR boxes i and ii was required for coactivation of PPAR $\gamma$ , consistent with the requirement of intact motifs i and ii for maximal PPAR $\gamma$  transactivation.

#### 4. X-Ray crystal structures

The biochemical studies outlined above clearly outline the LXXLL motifs of SRC coactivators as being critical to the interaction with and coactivation of nuclear

receptors. Further insight into the molecular basis of these interactions can be found in the recently solved crystal structures of several nuclear receptor LBDs with coactivator fragments containing NR boxes. In the structure of TR $\beta$ -LBD complexed with T3 and a 13-aa peptide encompassing NR box ii of GRIP1, the leucines of the  $\alpha$ -helical NR box make contacts with a hydrophobic groove consisting of residues from helices 3, 4, 5, and 12 of TR $\beta$  (Darimont et al., 1998). A single LXXLL peptide interacts with each monomer of the TR $\beta$  dimer. Mutagenesis confirms the importance of these receptor residues for *in vitro* binding of GRIP1 to TR $\beta$ . A very similar structure is observed with agonist-bound ER $\alpha$ -LBD complexed with a peptide comprising NR box ii of GRIP1 (Shiau et al., 1998). However, in the antagonist-bound ER $\alpha$ -LBD structure, helix 12 of the ER $\alpha$  is occluding the coactivator-binding site, consistent with the inability of SRC coactivators to bind antagonist-bound nuclear receptors. Most strikingly, a region of helix 12 contains an NR box-like sequence (LXXML) and functions as an intramolecular mimic of the LXXLL motif by making contact with the hydrophobic groove. This structural data supports the model of allosteric inhibition of the RXR–RAR heterodimer by the RXR AF-2 domain (Westin et al., 1998). These observations provide a molecular basis of antagonist function via conformational changes of helix 12 and inhibition of coactivator binding. Finally, the structure of the PPAR $\gamma$ -LBD bound to rosiglitazone and an 88-aa fragment of SRC-1 containing NR boxes i and ii has also been described (Nolte et al., 1998). This study details the function of a “charge clamp” of conserved glutamine and lysine residues of the LBD that positions the LXXLL motif into the hydrophobic groove of the receptor. In addition, the two NR boxes of the SRC-1 fragment are observed to contact simultaneously the PPAR $\gamma$  dimer, providing further support for the role of multiple motifs in mediating coactivator–receptor interactions.

### 5. Transcriptional activation by SRC coactivators

The SRC coactivators also contain an intrinsic transcriptional activation function, which is evident upon tethering coactivator to DNA via a heterologous DNA-binding domain. All three members are able to efficiently activate transcription when fused to the Gal4 DNA-binding domain in both yeast and mammalian cells (Li et al., 1997; Oñate et al., 1998; Voegel et al., 1996). Detailed deletion analysis has subsequently mapped the activation domain (AD) as being located C-terminal to the receptor-interacting domain. Interestingly, this AD also contains three additional LXXLL motifs representing NR boxes iv, v, and vi, that have been linked to interaction with the general transcriptional activators CBP/p300. CBP/p300 has been demonstrated to interact

with SRC proteins *in vitro* and *in vivo* and mutation of one or more of the AD NR boxes markedly impairs these interactions, as well as the activation function of the coactivator (Voegel et al., 1998; McInerney et al., 1998). Furthermore, microinjection studies have shown that anti-CBP antibodies abolish the ability of SRC-1 to coactivate RAR, suggesting that CBP/p300 is required for the coactivation function of SRC-1 (McInerney et al., 1998). However, it is worth noting that additional, CBP-independent transcriptional activation domains have also been attributed to members of the SRC family, supporting the existence of multiple mechanisms of transcriptional activation by coactivators (Ikeda et al., 1999; Ma et al., 1999; Voegel et al., 1998). The contribution of these multiple activation domains to overall coactivator function is not completely clear, but several studies suggest that coactivators may preferentially utilize specific ADs depending on the receptor or activation function (AF-1 vs. AF-2) that is mediating the response to hormone (Ma et al., 1999). In particular, the N-terminal AF-1 activation domain seems to be most critical to transcriptional activation by the androgen receptor (AR). SRC-1 and GRIP1 have been demonstrated to bind and coactivate the AF-1 domain of the AR (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999). These interactions are mediated by the C-terminus of the SRC coactivator, rather than the centrally located domain containing NR boxes i/ii/iii. Consistent with this observation, mutation of these motifs does not inhibit the ability of the coactivator to enhance transcriptional activation by the full-length AR, but only the isolated AF-2 domain (Alen et al., 1999; Bevan et al., 1999). This AF-1 interacting domain also lacks the CBP-interacting domain of the coactivator, thus coactivation of AF-1 likely occurs via mechanisms other than CBP recruitment, but through additional coactivators, such as CARM1 (see below), which also binds the C-terminus of GRIP1 (Chen et al., 1999a). Interestingly, the N-terminus of the AR also can interact with the C-terminal AF-2 domain and is required for both AF-2-induced transcription and SRC coactivation of AF-2 activity (Alen et al., 1999). These studies suggest that the AF-1 and AF-2 domains may synergize for complete AR activity and that the interaction of the two activation domains may result in recruitment of coactivator to the AR. Finally, SRC coactivators have also been demonstrated to enhance the AF-1 activity of the ER $\alpha$  in the presence of both estradiol and tamoxifen, suggesting that the partial agonism of tamoxifen occurs by coactivator recruitment to the AF-1 domain (Webb et al., 1998).

Another potential mechanism of transcriptional activation by SRC coactivators is histone acetylation. Hyperacetylated histones have long been linked to transcriptionally active chromatin, for acetylation leads to an unpacking of the condensed chromatin network, thereby facilitating the access of transcription factors to

target gene promoters. Accordingly, both CBP/p300 and the CBP/p300-associated factor P/CAF have been demonstrated to possess potent histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996), which is required for transcriptional activation by CBP (Martinez-Balbas et al., 1998). Both factors interact with SRC coactivators, as well as with nuclear receptors themselves, and enhance receptor transcriptional activation (Kamei et al., 1996; Blanco et al., 1998; Chakravarti et al., 1996). Therefore, recruitment of HAT-containing coactivators by the receptor may lead to a modulation of chromatin structure, thereby facilitating the access of either additional transcriptional activators, such as the DRIP/TRAP complex (see below), or the assembly of the pre-initiation complex, ultimately leading to transcriptional activation. Interactions between coactivators and the basal transcription machinery may also play a role in transcriptional activation, for both CBP/p300 (Kwok et al., 1994; Yuan et al., 1996) and SRC-1 (Ikeda et al., 1999; Takeshita et al., 1996) have been reported to interact with TBP and TFIIB. Interestingly, moderate HAT activity has also been attributed to SRC-1 and ACTR, suggesting that liganded nuclear receptors recruit a coactivator complex containing multiple enzymatic activities (Chen et al., 1997; Spencer et al., 1997). The apparent redundancy of HAT activities among the coactivator complex remains to be resolved completely. However, transcription factor-specific differences in HAT requirements have been established for RAR versus CREB via microinjection analysis (Korzus et al., 1998). Whereas P/CAF HAT activity was required for transcriptional activation by RAR, CBP HAT activity was required for CREB function. Additionally, cell-type and promoter-specific differences may also account for the existence of numerous HAT-containing coactivators. Finally, multiple HAT activities may be required if non-histone proteins also serve as substrates for these enzymes. In support of this, p300 has been demonstrated to acetylate p53, increasing its DNA binding activity (Gu and Roeder, 1997). Also, CBP and P/CAF can acetylate TFIIE and TFIIIF *in vitro*, which again links the basal transcription machinery to transcriptional activation by nuclear receptor coactivators (Imhof et al., 1997). Furthermore, a recent study reports that ACTR itself can be acetylated by CBP/p300 (Chen et al., 1999b). In this paper, hormone treatment results in enhanced histone acetylation at ER, RAR, and VDR target gene promoters and increased recruitment of coactivators, including ACTR and CBP/p300. However, this effect is transient in nature and is strongly downregulated after prolonged hormone treatment. Surprisingly, acetylation of ACTR by CBP/p300 at specific lysine residues causes the dissociation of ACTR from the DNA-bound ER homodimer. These results suggest that the mechanism of downregula-

tion of receptor activity involves release of the coactivator complex from the receptor via acetylation of the coactivator itself.

## 6. Diversity among SRC coactivators

One of the most important remaining questions to be answered concerning the function of SRC coactivators *in vivo* focuses on whether or not these three cofactors serve redundant functions. Although all three SRC family members do possess similar properties in terms of interactions with nuclear receptors and enhancement of transcriptional activation, several reports suggest that their activities are not completely overlapping and particularly outline a division between SRC-1 and TIF2/GRIP1 versus RAC3/ACTR/pCIP/AIB-1 functions. For example, microinjection of expression plasmids for SRC-1 or NCoA-2, but not pCIP, were able to rescue RAR-dependent activation in SRC-1 immunodepleted cells (Torchia et al., 1997). Also, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3/ACTR/AIB-1 is expressed at high levels in placenta, heart, and HeLa cells relative to TIF2 and SRC-1; thus it may serve a more prominent role in nuclear receptor function in these cells (Li and Chen, 1998). In addition, AIB-1 was cloned as a gene that is amplified in ER-positive BT-474, MCF-7, and ZR75 breast cancer cell lines (Anzick et al., 1997). AIB-1 mRNA and protein levels are expectedly higher in these cells as well. SRC-1 and TIF2/GRIP1 are expressed at relatively low levels in these cell lines, suggesting that AIB-1 is specifically involved in the pathogenesis of these tumors. Furthermore, the viability of an SRC-1 knockout mouse may, in part, be due to the observed compensatory overexpression of GRIP1/TIF2 in certain tissues (Xu et al., 1998). RAC3/pCIP levels are unchanged in these tissues compared to the wild-type mouse, again supporting a different functional role for this coactivator versus SRC-1 and TIF2/GRIP1. Finally, a recent study demonstrates that SRC-1 does not colocalize with ER $\alpha$  in rat mammary epithelial cells, but rather is expressed in a distinct subset of cells, suggesting that TIF2/GRIP1 or RAC3/ACTR/AIB-1 may be more important for ER $\alpha$  function in these cells (Shim et al., 1999).

## 7. Other nuclear receptor coactivators

In addition to the SRC family of coactivators discussed above, many other cofactors have been identified which stimulate the activity of nuclear receptors. For the sake of brevity, we will focus on those associated with SRC coactivators.

### 7.1. SRA

Recently, in a search for nuclear receptor cofactors, a novel steroid receptor RNA activator (SRA) was isolated in a yeast-two-hybrid screen using the AF-1 domain of the PR as the bait (Lanz et al., 1999). This coactivator is selective for the N-terminal AF-1 activation domain of nuclear receptors and can reverse estrogen-induced squelching of PR-driven gene expression. Interestingly, SRA is also selective for steroid receptors versus RXR heterodimers, for it enhances only PR, GR, AR, and ER activities while having no effect on the activities of the TR $\beta$ , RAR $\gamma$ , RXR $\gamma$ , or PPAR $\gamma$ . However, maybe the most surprising characteristic of SRA is that it apparently functions as an RNA transcript, which is evident from several observations. First, efforts to generate SRA-encoded protein *in vitro* or *in vivo* were not successful. Second, several mutant constructs of SRA that disrupt translational start sites or open reading frames were still able to potentiate PR activity. Third, SRA retained coactivation activity in the absence of protein synthesis via cyclohexamide treatment. Finally, SRA transcripts were identified as components of an SRC-1 complex *in vivo* via whole-cell fractionation followed by gel filtration chromatography. SRA, detected by RT-PCR, specifically copurified with SRC-1 in the same fractions. SRA mRNA was also efficiently coimmunoprecipitated with SRC-1 antibodies, further supporting the existence of a complex containing SRA and SRC-1. This study suggests that SRA is a novel RNA coactivator that forms a complex with SRC-1 *in vivo* and selectively enhances the activity of steroid hormone receptors via the AF-1 domain.

### 7.2. CARM1

A novel enzymatic activity was attributed to nuclear receptor coactivators with the cloning of CARM1 (coactivator-associated arginine methyltransferase 1) via a yeast-two-hybrid screen using the C-terminal amino acids 1121–1462 of GRIP1 (Chen et al., 1999a). This region represents a second, CBP-independent activation domain of GRIP1 (Ma et al., 1999). CARM1 showed extensive homology to the PRMT (protein methyltransferase) family of arginine-specific methyltransferases and interacted with all three members of the SRC coactivator family *in vitro*. Furthermore, CARM1 contained potent histone methyltransferase activity *in vitro*, with a preference for histone H3. *In vivo*, CARM1 enhanced transcription by a Gal4-DBD fusion of GRIP1 1121–1462 and further stimulated GRIP1 coactivation of AR, TR, and ER activities. This coactivation function was dependent upon three amino acids located in the region critical to methyltransferase activity, suggesting that this enzymatic activity is required for CARM1's ability to enhance receptor function. In the

absence of GRIP1, CARM1 had no effect on receptor function, thus SRC coactivators are likely required in order to recruit CARM1 to the receptor complex. Overall, the cloning of CARM1 contributes to the mechanism by which SRC coactivators activate transcription through multiple domains. One activation domain may be required in order to recruit CBP/p300 and histone acetylation activity, while the second activation domain recruits CARM1 and histone methylation activity. These multiple enzymatic functions may be promoter specific or cooperate to remodel chromatin and facilitate transcriptional activation.

### 7.3. PGC-1

The cloning and characterization of PGC-1 (PPAR gamma coactivator-1) was a critical finding to the field of nuclear receptor coactivators, for it linked coactivator function to the regulation of a specific physiological process, namely adaptive thermogenesis. PGC-1 was isolated in a yeast-two-hybrid screen using PPAR $\gamma$  183–505 as the bait and was demonstrated to interact with several members of the nuclear receptor superfamily (Puigserver et al., 1998). It also possesses potent coactivation function for PPAR $\gamma$  and TR activities at the UCP-1 (uncoupling protein-1) promoter, inducing the expression of this mitochondrial protein involved in heat generation in brown fat cells. Consistently, PGC-1 is also upregulated in muscle and brown fat cells upon exposure to cold temperatures. Further studies have demonstrated that PGC-1 enhances mitochondrial biogenesis and oxygen consumption in muscle cells via the induction of UCP-2 and the regulation of NRFs (nuclear respiratory factors), which are transcription factors that regulate genes involved in mitochondrial DNA replication and transcription (Wu et al., 1999). Finally, a very recent study reports the functional association between PGC-1 and SRC-1 (Puigserver et al., 1999). SRC-1, as well as CBP/p300, interacts with PGC-1 *in vitro* and *in vivo* and enhances transcriptional activation by a Gal4DBD fusion of PGC-1 in transient transfection assays. These interactions are mediated by SRC-1 782–1139 and p300 1805–2441. Intriguingly, expression of PPAR $\gamma$  or NRF-1 also enhanced Gal-PGC-1 activity, while cotransfection of PPAR $\gamma$  increased the interaction between PGC-1 and SRC-1 or CBP/p300 *in vitro* and *in vivo*. These data support a model of PGC-1 activation in which the interaction with a transcription factor such as PPAR $\gamma$  stimulates PGC-1 activity by inducing SRC-1 recruitment (Puigserver et al., 1999). This recruitment likely is the result of a conformational change in PGC-1 that occurs upon binding to the transcription factor. This study also suggests the SRC coactivator function may be essential to adaptive thermogenesis, for it may be required for transcriptional activation by PGC-1.

## 8. DRIP/TRAP coactivator complexes

Much effort has been made recently to isolate and purify an entire complex of polypeptides that functions to coactivate nuclear receptor function. To this end, several groups have identified virtually identical complexes that appear distinct from the SRC coactivator complex. Using the VDR-LBD as an affinity matrix, a complex was purified from Namalwa cell extracts termed DRIP (VDR-interacting proteins) that specifically interacts with ligand-bound VDR-LBD (Rachez et al., 1998). The same complex was also purified using affinity chromatography from HeLa cells that constitutively express Flag-tagged TR and named TRAP (TR-associated proteins) (Fondell et al., 1996). Subsequently, this complex was identified as ARC (activator-recruited cofactor) (Naar et al., 1999) and SMCC (Srb/Mediator coactivator complex) (Gu et al., 1999). The DRIP/TRAP complex lacks CBP/p300 or SRC proteins (Rachez et al., 1999) and is recruited to the receptor AF-2 domain by the DRIP205/TRAP220 subunit via a single LXXLL motif (Rachez et al., 1999; Yuan et al., 1998). Unlike SRC coactivators, the DRIPs/TRAPs have been demonstrated to be required

for transcriptional activation by nuclear receptors in cell-free *in vitro* transcription assays (Rachez et al., 1998; Fondell et al., 1996). The DRIPs also enhance VDR activity on chromatin-organized templates despite a lack of HAT activity, suggesting a potential unidentified chromatin remodeling function (Rachez et al., 1999). Furthermore, it is evident that this complex plays a more global role in transcriptional activation rather than being specific to nuclear receptors, for ARC was identified as a coactivator for VP16 and p53 (Naar et al., 1999), while SMCC enhances p53 activity (Gu et al., 1999). What is not clear is the mechanism by which the DRIP/TRAP and SRC complexes both contribute to overall nuclear receptor function. One possibility involves a two-step model in which the SRC complex is first recruited to the nuclear receptor to open up the chromatin network via histone acetylation (Freedman, 1999). This would allow access for the large DRIP/TRAP complex, which would subsequently remodel chromatin, facilitating the organization of the pre-initiation complex or binding of other transcription factors. However, it is also possible that the DRIPs/TRAPs may target RNA polymerase to the target gene promoter, for several subunits are homolo-

## Model of SRC coactivator function

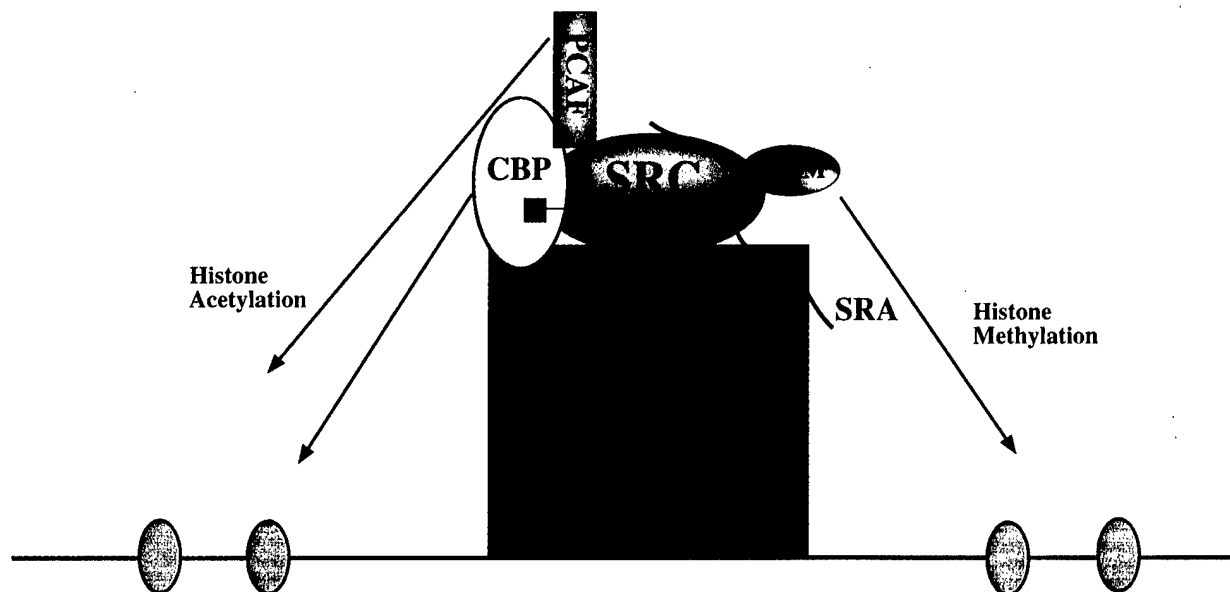


Fig. 2. Model of SRC coactivator function. The nuclear receptor is able to recruit an SRC coactivator upon binding hormone, which subsequently results in the recruitment of additional coactivators to the complex. SRC is able to interact with the receptor and CBP/p300 via LXXLL nuclear receptor (NR) boxes. The histone acetylation and methylation activities of various constituents of the coactivator complex facilitate the relaxation of the chromatin architecture at the target gene promoter, thereby enhancing transcriptional activation. It should be noted that this is only a general model of the coactivator complex. It is likely that additional cofactors are involved and that different receptors may recruit different components of the complex, thus achieving a level of specificity among receptors and coactivators. NR = nuclear receptor, SRC = steroid receptor coactivator, SRA = steroid receptor RNA activator, CARM = coactivator associated arginine methyltransferase, CBP = CREB-binding protein, PCAF = p300/CBP-associated factor.

gous to proteins found in Mediator, a transcriptional regulatory complex that associates with RNA pol II (Kim et al., 1994). In support of this, RNA pol II can be isolated with the SMCC complex at low ionic strength (Gu et al., 1999). One also cannot rule out the possibility that SRC and DRIP/TRAP functions are not integrated at all, but rather have cell-type, promoter, or transcription factor specificity. Specificity may also be the result of the alteration of one or more of the subunits of the complex, depending on the target gene. Overall, it is clear that the DRIP/TRAP complex is likely involved in the regulation of a broad range of signaling pathways, but whose biological role is unknown.

## 9. Model of SRC function

In order to integrate the wealth of data collected on the mechanism of action of SRC coactivators, we propose the following model of SRC function in the regulation of nuclear receptor activity (Fig. 2). Hormone binding triggers nuclear translocation of Type I steroid receptors and the release of the corepressor complex from Type II non-steroid receptors and subsequent recruitment of an SRC coactivator to the target gene promoter. SRC is able to interact with the AF-2 domain of each monomer of the dimer via multiple,  $\alpha$ -helical NR boxes located in the receptor-interacting domain. SRC is likely complexed with the RNA coactivator SRA, which enhances AF-1 activity. After initial SRC docking to the receptor, it is able to recruit additional coactivators to the complex. These include CBP/p300, which uses the NR boxes of the SRC transcriptional activation domain for interaction with coactivator, and the CBP/p300-associated factor P/CAF. Additional, direct interactions between CBP/p300 and nuclear receptors and between P/CAF and SRC have also been reported, which may enhance complex formation. Furthermore, SRC is also able to recruit CARM1 to the target gene via a different domain than that required for CBP/p300 binding. Once this complex is assembled, the histone acetylase activities of CBP/p300, P/CAF, and possibly SRC itself, together with the histone methyltransferase activity of CARM1, serve to remodel the chromatin architecture, thus facilitating the access of additional transcription factors, coactivators such as the DRIP/TRAP complex, and/or the basal transcription machinery to the target gene promoter to activate transcription. Of course, caveats to this model likely exist. For example, the coactivator complex may be comprised of different components depending on the specific nuclear receptor, cell type, or target gene. Different coactivator complex components may create a level of specificity among different receptors that answers the questions surrounding the potential redundancy among the members of the SRC family. Also, with the

intense focus on hormone action and plethora of receptor cofactors being cloned in recent years, it is likely that additional members of the coactivator complex have yet to be identified. In addition, non-histone substrates for the enzymatically active cofactors may be involved, for as described above, CBP/p300 can acetylate non-histone proteins such as ACTR, p53, and TFIIE/TFIIF. Finally, it is possible that the receptor is able to recruit single, pre-formed coactivator complex to the target gene upon hormone binding. However, though the precise details of transcriptional activation by nuclear receptors are still not clear, it is evident that the SRC family of coactivators is critical to receptor function and will continue to warrant investigation into its role in intracellular signaling pathways.

## Acknowledgements

This publication was made possible by grant number DK52888 from the NIH (J.D.C.) and a USAMRMC pre-doctoral fellowship (C.L.). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or USAMRMC.

## References

- Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., Peeters, B., 1999. The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol. Cell Biol.* 19, 6085–6097.
- Alland, L., Muhle, R., Hou Jr., H., Potes, J., Chin, L., Schreiber-Agus, N., DePino, R.A., 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression see comments. *Nature* 387, 49–55.
- Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M., Meltzer, P.S., 1997. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277, 965–968.
- Bannister, A.J., Kouzarides, T., 1996. The CBP co-activator is a histone acetyltransferase. *Nature* 384, 641–643.
- Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M., Parker, M.G., 1999. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. *Mol. Cell Biol.* 19, 8383–8392.
- Blanco, J.C., Minucci, S., Lu, J., Yang, X.J., Walker, K.K., Chen, H., Evans, R.M., Nakatani, Y., Ozato, K., 1998. The histone acetylase PCAF is a nuclear receptor coactivator. *Genes Dev.* 12, 1638–1651.
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., Moras, D., 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR- $\alpha$  see comments. *Nature* 375, 377–382.
- Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389, 753–758.
- Carapeti, M., Aguiar, R.C., Goldman, J.M., Cross, N.C., 1998. A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* 91, 3127–3133.
- Chakravarti, D., LaMorte, V.J., Nelson, M.C., Nakajima, T., Schulman, I.G., Juguilon, H., Montminy, M., Evans, R.M., 1996. Role of CBP/P300 in nuclear receptor signalling. *Nature* 383, 99–103.

- Chang, C., Norris, J.D., Gron, H., Paige, L.A., Hamilton, P.T., Kenan, D.J., Fowlkes, D., McDonnell, D.P., 1999. Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries discovery of peptide antagonists of estrogen receptors alpha and beta. *Mol. Cell Biol.* 19, 8226–8239.
- Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., Stallcup, M.R., 1999a. Regulation of transcription by a protein methyltransferase. *Science* 284, 2174–2177.
- Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y., Evans, R.M., 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90, 569–580.
- Chen, H., Lin, R.J., Xie, W., Wilpitz, D., Evans, R.M., 1999b. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase in process citation. *Cell* 98, 675–686.
- Chen, J.D., Evans, R.M., 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors see comments. *Nature* 377, 454–457.
- Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., Yamamoto, K.R., 1998. Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev.* 12, 3343–3356.
- Ding, X.F., Anderson, C.M., Ma, H., Hong, H., Uht, R.M., Kushner, P.J., Stallcup, M.R., 1998. Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. *Mol. Endocrinol.* 12, 302–313.
- Feng, W., Ribeiro, R.C., Wagner, R.L., Nguyen, H., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., Kushner, P.J., West, B.L., 1998. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 280, 1747–1749.
- Fondell, J.D., Ge, H., Roeder, R.G., 1996. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc. Natl. Acad. Sci. USA* 93, 8329–8333.
- Freedman, L.P., 1999. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell* 97, 5–8.
- Gu, W., Malik, S., Ito, M., Yuan, C.X., Fondell, J.D., Zhang, X., Martinez, E., Qin, J., Roeder, R.G., 1999. A novel human SRB/MED-containing cofactor complex SMCC involved in transcription regulation. *Cell* 3, 97–108. published erratum appears in *Mol. Cell* 3 (4) 1999, following 541.
- Gu, W., Roeder, R.G., 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90, 595–606.
- Heery, D.M., Kalkhoven, E., Hoare, S., Parker, M.G., 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387, 733–736.
- Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenman, R.N., Rose, D.W., Glass, C.K., Rosenfeld, M.G., 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression see comments. *Nature* 387, 43–48.
- Hong, H., Kohli, K., Trivedi, A., Johnson, D.L., Stallcup, M.R., 1996. GRIP1 a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc. Natl. Acad. Sci. USA* 93, 4948–4952.
- Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamci, Y., Soderstrom, M., Glass, C.K., Rosenfeld, M.G., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor see comments. *Nature* 377, 397–404.
- Ikeda, M., Kawaguchi, A., Takeshita, A., Chin, W.W., Endo, T., Onaya, T., 1999. CBP-dependent and independent enhancing activity of steroid receptor coactivator-1 in thyroid hormone receptor-mediated transactivation. *Mol. Cell Endocrinol.* 147, 103–112.
- Imhof, A., Yang, X.J., Ogryzko, V.V., Nakatani, Y., Wolffe, A.P., Ge, H., 1997. Acetylation of general transcription factors by histone acetyltransferases. *Curr. Biol.* 7, 689–692.
- Kalkhoven, E., Valentine, J.E., Heery, D.M., Parker, M.G., 1998. Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. *Embo J.* 17, 232–243.
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K., Rosenfeld, M.G., 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85, 403–414.
- Kim, Y.J., Bjorklund, S., Li, Y., Sayre, M.H., Kornberg, R.D., 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* 77, 599–608.
- Korzus, E., Torchia, J., Rose, D.W., Xu, L., Kurokawa, R., McInerney, E.M., Mullen, T.M., Glass, C.K., Rosenfeld, M.G., 1998. Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science* 279, 703–707.
- Kwok, R.P., Lundblad, J.R., Chivria, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., Goodman, R.H., 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB see comments. *Nature* 370, 223–226.
- Lanz, R.B., McKenna, N.J., Oñate, S.A., Albrecht, U., Wong, J., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1999. A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. *Cell* 97, 17–27.
- Lee, S.K., Kim, H.J., Na, S.Y., Kim, T.S., Choi, H.S., Im, S.Y., Lee, J.W., 1998. Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J. Biol. Chem.* 273, 16651–16654.
- Leers, J., Treuter, E., Gustafsson, J.A., 1998. Mechanistic principles in NR box-dependent interaction between nuclear hormone receptors and the coactivator TIF2. *Mol. Cell Biol.* 18, 6001–6013.
- Li, H., Chen, J.D., 1998. The receptor-associated coactivator 3 activates transcription through CREB-binding protein recruitment and autoregulation. *J. Biol. Chem.* 273, 5948–5954.
- Li, H., Gomes, P.J., Chen, J.D., 1997. RAC3 a steroid/nuclear receptor-associated coactivator that is related to SRC1 and TIF2. *Proc. Natl. Acad. Sci. USA* 94, 8479–8484.
- Ma, H., Hong, H., Huang, S.M., Irvine, R.A., Webb, P., Kushner, P.J., Coetzee, G.A., Stallcup, M.R., 1999. Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins in process citation. *Mol. Cell Biol.* 19, 6164–6173.
- Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily the second decade. *Cell* 83, 835–839.
- Martinez-Balbas, M.A., Bannister, A.J., Martin, K., Haus-Seuffert, P., Meisterernst, M., Kouzarides, T., 1998. The acetyltransferase activity of CBP stimulates transcription. *Embo J.* 17, 2886–2893.
- McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., Milburn, M.V., Glass, C.K., Rosenfeld, M.G., 1998. Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. *Genes Dev.* 12, 3357–3368.
- McKenna, N.J., Lanz, R.B., O'Malley, B.W., 1999. Nuclear receptor coregulators: cellular and molecular biology. *Endocrinol. Rev.* 20, 321–344.
- Misiti, S., Schomburg, L., Yen, P.M., Chin, W.W., 1998. Expression and hormonal regulation of coactivator and corepressor genes. *Endocrinology* 139, 2493–2500.
- Murre, C., McCaw, P.S., Baltimore, D., 1989a. A new DNA binding

- and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56, 777–883.
- Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., Weintraub, H., Baltimore, D., 1989b. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* 58, 537–544.
- Na, S.Y., Lee, S.K., Han, S.J., Choi, H.S., Im, S.Y., Lee, J.W., 1998. Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated transactivations. *J. Biol. Chem.* 273, 10831–10834.
- Naar, A.M., Beaurang, P.A., Zhou, S., Abraham, S., Solomon, W., Tjian, R., 1999. Composite co-activator ARC mediates chromatin-directed transcriptional activation. *Nature* 398, 828–832.
- Nagy, L., Kao, H.Y., Chakravarti, D., Lin, R.J., Hassig, C.A., Ayer, D.E., Schreiber, S.L., Evans, R.M., 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 89, 373–380.
- Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K., Milburn, M.V., 1998. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* 395, 137–143.
- Norris, J.D., Paige, L.A., Christensen, D.J., Chang, C.Y., Huacani, M.R., Fan, D., Hamilton, P.T., Fowlkes, D.M., McDonnell, D.P., 1999. Peptide antagonists of the human estrogen receptor. *Science* 285, 744–746.
- Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., Nakatani, Y., 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87, 953–959.
- Oñate, S.A., Boonyaratankornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P., O'Malley, B.W., 1998. The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.* 273, 12101–12108.
- Oñate, S.A., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1995. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270, 1354–1357.
- Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B., Spiegelman, B.M., 1999. Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* 286, 1368–1371.
- Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., Spiegelman, B.M., 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92, 829–839.
- Rachez, C., Lemon, B.D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A.M., Erdjument-Bromage, H., Tempst, P., Freedman, L.P., 1999. Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* 398, 824–828.
- Rachez, C., Suldan, Z., Ward, J., Chang, C.P., Burakov, D., Erdjument-Bromage, H., Tempst, P., Freedman, L.P., 1998. A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. *Genes Dev.* 12, 1787–1800.
- Renaud, J.P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., Moras, D., 1995. Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature* 378, 681–689.
- Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L., 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927–937.
- Shim, W.S., DiRenzo, J., DeCaprio, J.A., Santen, R.J., Brown, M., Jeng, M.H., 1999. Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. *Proc. Natl. Acad. Sci. USA* 96, 208–213.
- Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Oñate, S.A., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389, 194–198.
- Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C.S., Chin, W.W., 1997. TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. *J. Biol. Chem.* 272, 27629–27634.
- Takeshita, A., Yen, P.M., Misiti, S., Cardona, G.R., Liu, Y., Chin, W.W., 1996. Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. *Endocrinology* 137, 3594–3597.
- Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., Rosenfeld, M.G., 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function see comments. *Nature* 387, 677–684.
- Tsai, M.J., O'Malley, B.W., 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 63, 451–486.
- Voegel, J.J., Heine, M.J., Tini, M., Vivat, V., Chambon, P., Gronemeyer, H., 1998. The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. *EMBO J.* 17, 507–519.
- Voegel, J.J., Heine, M.J.S., Zechel, C., Chambon, P., Gronemeyer, H., 1996. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* 15, 3667–3675.
- Wagner, R.L., Apriletti, J.W., West, B.L., Baxter, J.D., Fletterick, R.J., 1995. A structural role for hormone in the thyroid hormone receptor ligand-binding domain. *Nature* 378, 690–697.
- Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E., Katzenellenbogen, B.S., Stallcup, M.R., Kushner, P.J., 1998. Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Mol. Endocrinol.* 12, 1605–1618.
- Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G., Glass, C.K., 1998. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature* 395, 199–202.
- Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C., Spiegelman, B.M., 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98, 115–124.
- Xu, J., Qiu, Y., DeMayo, F.J., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1998. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* 279, 1922–1925.
- Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K., Kato, S., 1999. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science* 283, 1317–1321.
- Yang, X.J., Ogryzko, V.V., Nishikawa, J., Howard, B.H., Nakatani, Y., 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* 382, 319–324.
- Yuan, W., Condorelli, G., Caruso, M., Felsani, A., Giordino, A., 1996. Human p300 protein is a coactivator for the transcription factor MyoD. *J. Biol. Chem.* 271, 9009–9013.
- Yuan, C.X., Ito, M., Fondell, J.D., Fu, Z.Y., Roeder, R.G., 1998. The TRAP220 component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc. Natl. Acad. Sci. USA* 95, 7939–7944.



## Differential Mechanisms of Nuclear Receptor Regulation by Receptor-associated Coactivator 3\*

(Received for publication, October 27, 1999, and in revised form, December 6, 1999)

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Steroid and nuclear receptor coactivators (NCoAs) have been implicated in the regulation of nuclear receptor function by enhancing ligand-dependent transcriptional activation of target gene expression. We have previously isolated receptor-associated coactivator 3 (RAC3), which belongs to the steroid receptor coactivator family. In this study, we investigated the differential mechanisms by which RAC3 interacts with and modulates the transcriptional activity of different nuclear receptors. We found that the vitamin D receptor (VDR) and estrogen receptor  $\beta$  interact with different  $\alpha$ -helical LXXLL motifs of RAC3. Peptides corresponding to these motifs have diverse affinities for the VDR and estrogen receptor  $\beta$ , and mutation of specific motifs differentially impairs the ability of RAC3 to interact with these receptors *in vitro*. Consequently, these mutations inhibit the enhancement of transcriptional activation by these receptors *in vivo*. Furthermore, we found that the activation function-2 (AF-2) domain of the retinoid X receptor interferes with RAC3 binding to a DNA-bound VDR/retinoid X receptor (RXR) heterodimer, whereas the VDR AF-2 domain is required for this interaction. These results suggest a receptor-specific binding preference for the different LXXLL motifs of RAC3, which may provide flexibility for RAC3 to differentially regulate the function of different nuclear receptors.

The vitamin D receptor (VDR)<sup>1</sup> and estrogen receptor  $\beta$  (ER $\beta$ ) belong to the steroid/thyroid hormone receptor superfamily, which is a large class of ligand-dependent transcription factors that plays critical roles in regulating genes involved in a wide array of biological processes, including development and homeostasis (1). This superfamily can be divided into three subgroups. The ER $\beta$  is a Type I receptor, which also includes receptors for steroids such as progestins, androgens, glucocorticoids, and mineralcorticoids. These receptors are coupled to

heat shock proteins and sequestered to the cytoplasm in the absence of ligand. Upon hormone binding, they dissociate from the heat shock proteins, homodimerize, and translocate to the nucleus where they bind to cognate response elements consisting of palindromic repeats. The VDR is a Type II receptor like those for thyroid hormone (TR) and all-trans retinoic acid (RAR). These receptors are strictly nuclear and form heterodimers with the receptor for 9-cis retinoic acid (RXR). They also bind constitutively to response elements consisting of direct repeats (DRs). A third class of nuclear receptors is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

Most members of the nuclear receptor superfamily share a common domain structure. The N terminus contains the variable A/B region, which also includes the ligand-independent AF-1 activation domain. The highly conserved DNA binding domain and the C-terminal ligand binding domain (LBD) follow this region. The LBD contains the ligand-dependent AF-2 activation domain and also mediates dimerization of nuclear receptors. In the absence of ligand, nuclear receptors are able to repress basal transcription via functional interactions with the nuclear receptor corepressors SMRT and NCoR (2, 3). SMRT and NCoR are found in complexes with the corepressor mSin3 and the histone deacetylase HDAC1, suggesting that transcriptional repression by nuclear receptors may involve histone deacetylation (4–6). Ligand binding triggers the release of these corepressors and subsequent recruitment of coactivators through a drastic conformational change in the AF-2 domain of the receptor. Structural studies have demonstrated that helix 12, which contains the AF-2 domain, projects away from the LBD in the unbound RXR structure, but rotates nearly 180° to pack tightly against the LBD upon hormone binding in the RAR, TR, and ER (7–10). This conformational change, together with induced changes in helices 3–5, is believed to facilitate interactions of the receptor with coactivators (11–16).

Coactivators recruited by ligand-bound nuclear receptors include members of the SRC family of coactivators such as SRC1 (also known as NCoA-1), TIF2/GRIP1 (also known as SRC2 or NCoA-2), and RAC3/ACTR/pCIP/AIB1 (also known as SRC3 or NCoA-3) (reviewed in Refs. 17 and 18). SRC family members share an N-terminal basic helix-loop-helix/PAS-A/PAS-B domain of unknown function, centrally located receptor interaction domain, and C-terminal transcriptional activation domain. These cofactors interact with receptors in a hormone- and AF-2-dependent manner and enhance transcriptional activation by nuclear receptors. Both coactivators and receptors also have been demonstrated to interact with the general transcriptional activators CBP/p300 and PCAF (19–26), suggesting that a large multi-protein complex is assembled at the target gene promoter to activate transcription. Furthermore, several coactivators, including SRC1, ACTR, PCAF, and CBP/p300, possess

\* This work was supported by National Institutes of Health Grant 1 R01 DK52888-01 (to J. D. C.) and a U. S. Army Medical Research and Material Command pre-doctoral fellowship (to C. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: VDR, vitamin D receptor; RXR, retinoid X receptor; RAR, retinoic acid receptor; ER $\beta$ , estrogen receptor  $\beta$ ; RAC3, receptor-associated coactivator 3; SRC, steroid receptor coactivator; RID, receptor-interacting domain; AF-2, activation function-2 domain; NR box, nuclear receptor interacting box; DR, direct repeat; LBD, ligand binding domain; GST, glutathione S-transferase; NCoA, nuclear receptor coactivator; PAS, Per-Arnt-Sim domain; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; ERE, estrogen response element; TR, thyroid hormone.

intrinsic histone acetylation activity, which disrupts nucleosomes (21, 27–30). Therefore, the mechanism by which nuclear receptors activate transcription may entail the recruitment of a coactivator complex via the AF-2 domain that can modify chromatin structure, thereby facilitating access to the promoter by the general transcription machinery.

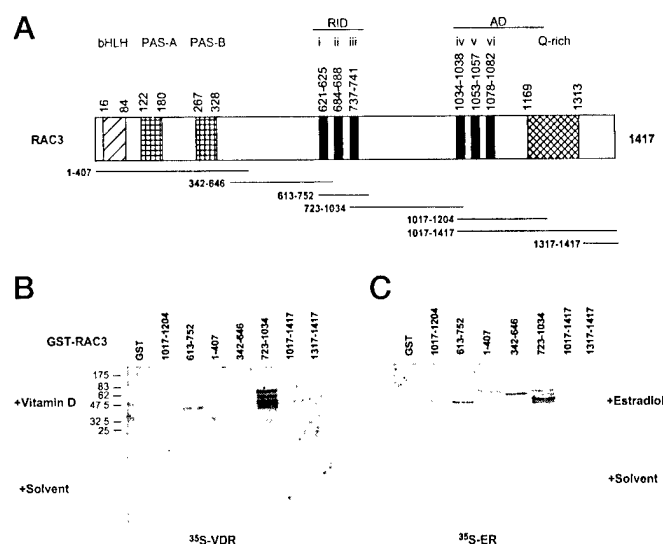
Intriguingly, members of the SRC family of coactivators have been found to contain several conserved motifs, termed NR boxes, with the consensus sequence LXXLL, where X is any amino acid (31). Motifs within the receptor-interacting domain and transcriptional activation domains of SRC1 and TIF2 have been demonstrated to mediate interactions with liganded nuclear receptors and CBP/p300, respectively (23, 32). Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic  $\alpha$ -helices with the leucine residues comprising a hydrophobic surface on one face of the helix (11, 12, 14, 24). The helical motif is able to interact with the AF-2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding (11, 14, 16). Mutational analyses of the NR boxes of SRC1 and TIF2/GRIP1 have also uncovered a receptor-specific code of interaction, where different nuclear receptors require different NR boxes to interact with the coactivator (32–34). These studies indicate that flanking residues outside the NR box may also be important to nuclear receptor-coactivator interactions.

In this study, we investigate the mechanisms by which RAC3 regulates the function of the VDR and ER $\beta$ , for little is known concerning the regulation of these receptors by SRC coactivators, particularly RAC3. These analyses reveal receptor-specific interactions in which the VDR and ER $\beta$  interact with different surfaces of RAC3. We demonstrate different preferences of these receptors for specific NR boxes of RAC3 and that single mutations in these LXXLL motifs are able to severely impair the ability of RAC3 to interact with and, thus, coactivate the VDR and ER $\beta$ . In analyzing the requirement of nuclear receptor AF-2 domains, we observe that the AF-2 domain of RXR can inhibit RAC3-RID binding to the DNA-bound VDR/RXR heterodimer, whereas the AF-2 domain of VDR is required for this interaction. These data add a new level of complexity to the regulation of nuclear receptor activity by SRC coactivators and suggest that different classes of nuclear receptors may be regulated by RAC3 via different mechanisms.

#### EXPERIMENTAL PROCEDURES

**Far Western Analysis**—Far Western assays were carried out as described (20). Briefly, GST fusion proteins were expressed in DH5 $\alpha$  cells and purified with glutathione-agarose beads (Amersham Pharmacia Biotech). Purified proteins were then separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Proteins were denatured with 6 M guanidine hydrochloride and renatured by the stepwise dilution of guanidine hydrochloride. Membranes were then blocked and hybridized overnight with  $^{35}$ S-labeled protein. The membrane was washed, and bound probe was detected by autoradiography.  $^{35}$ S-labeled probes were generated by Quick-coupled *in vitro* transcription/translation (Promega). For peptide competition experiments, the given concentration of peptide was added to the probe 10 min before hybridization with the membrane. Peptide sequences were as follows: NR box i peptide (LESKGHKKLLQLLTSSDDRGHSSL), NR box ii peptide (LQEKHRLHKKLLQNGNSP), NR box iii peptide (KKKENNALLRYLLDRDD), control peptide (GSGSATATLYENKPRP-PYIL). Radioactive bands were quantified by PhosphorImager using the ImageQuant software (Molecular Dynamics).

**GST Pull-down Assay**—Approximately 5  $\mu$ g of purified GST fusion protein was incubated with 5  $\mu$ l of  $^{35}$ S-labeled protein with moderate shaking at 4 °C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 0.05% Nonidet P-40, 1 mM dithiothreitol, 1 mg/ml BSA). The bound protein was washed three times with binding buffer, and beads were collected by centrifugation. The



**FIG. 1. The VDR and ER $\beta$  interact with different fragments of RAC3.** A, schematic illustration of RAC3 and its functional domains as well as the purified GST-RAC3 fragments used for Far Western assays. AD, activation domain; i–vi, RAC3 LXXLL NR boxes. bHLH, basic helix-loop-helix. B, Far Western assay using  $^{35}$ S-VDR to probe GST-RAC3 fusion proteins in the presence (top) or absence (bottom) of 1  $\mu$ M 1,25-dihydroxy-vitamin D<sub>3</sub>. C, Far Western assay using  $^{35}$ S-ER $\beta$  to probe GST-RAC3 fusion proteins in the presence (top) or absence (bottom) of 1  $\mu$ M 17 $\beta$ -estradiol.

bound protein was eluted in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and detected by autoradiography.

**Site-directed Mutagenesis**—NR box mutants were generated with the Quick-change site-directed mutagenesis system (Stratagene). The sequences of all mutant constructs were confirmed by dideoxynucleotide chain termination reactions using the T7 Sequenase protocol (U. S. Biochemical Corp.).

**Gel Electrophoresis Mobility Shift Assay**—The sequence of the DR3 element used for VDR/RXR gel-shift assays is AGCTTAAGAGGTCAGAAAGGTCACCTCGCAT. The double-stranded DR3 was end-labeled with [ $^{32}$ P]dCTP by standard Klenow fill-in reaction. The purified probe was incubated with  $^{35}$ S-labeled receptors in binding buffer containing 7.5% glycerol, 20 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1  $\mu$ g of poly(dI-dC) and 100 mM KCl. Wild-type or mutant GST-RAC3-RID was eluted from glutathione-agarose beads with 10 mM reduced glutathione and added to the binding reaction. The DNA-protein complex was formed on ice for 1 h and resolved on a 5% native polyacrylamide gel, which was subsequently dried and subjected to autoradiography.

**Cell Culture and Transient Transfection**—HEK293 and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5  $\mu$ g/ $\mu$ l gentamycin at 37 °C, 5% CO<sub>2</sub>. Cells were plated for transfection in Dulbecco's modified Eagle's medium supplemented with 10% resin charcoal-stripped fetal bovine serum in 12- or 6-well plates 1 day before transfection. HEK293 cells were transfected using the standard calcium phosphate method, whereas CV-1 cells were transfected using LipofectAMINE according to the manufacturer's protocol (Life Technologies, Inc.). Twelve hours after transfection, cells were washed with phosphate-buffered saline and refed fresh medium containing the indicated concentration of ligand. After 24 h, cells were harvested for  $\beta$ -galactosidase and luciferase activities as described (35). Luciferase activity was determined with a MLX plate luminometer (Dynex) and normalized relative to  $\beta$ -galactosidase activity.

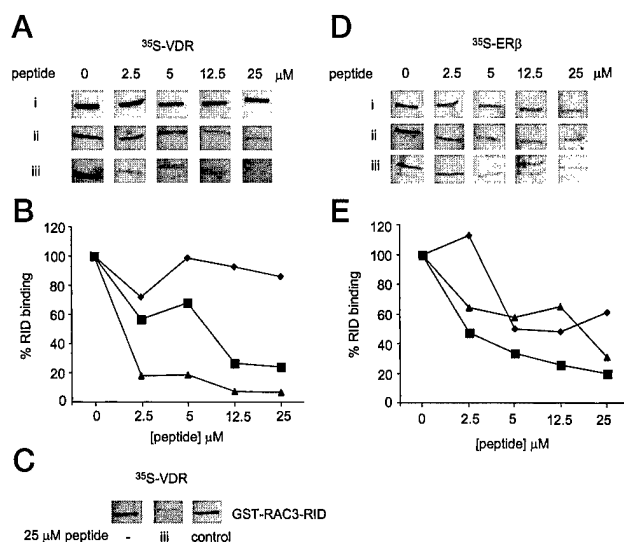
#### RESULTS

**VDR and ER $\beta$  Interact with Multiple Surfaces of RAC3**—We have previously defined the minimal receptor interacting domain (RID) of RAC3 to be amino acids 613–752, which contains the first three LXXLL motifs (Fig. 1A) (20). We wished to further determine if different receptors were capable of binding to the same regions of RAC3. To accomplish this, we purified a panel of GST-RAC3 fusion proteins, in total comprising the full-length RAC3 (Fig. 1A), and probed these fusions with

[ $^{35}$ S]methionine-labeled VDR and ER $\beta$  in a Far Western assay. The VDR, as expected, interacted in a ligand-dependent manner with GST-RAC3 613–752 in this assay (Fig. 1B). It also bound GST-RAC3 723–1034, which only contained NR box iii, in a ligand-dependent manner. The VDR did not interact with any other GST-RAC3 fragment, including GST-RAC3 342–646, which contained NR box i. It also appeared that the VDR interacted more strongly with GST-RAC3 723–1034 than with GST-RAC3 613–752, suggesting a more important role for NR box iii in the RAC3-VDR interaction. However, a different pattern was evident upon repeating this assay with  $^{35}$ S-ER $\beta$ , for in addition to ligand-dependent interactions with GST-RAC3 613–752 and 723–1034, ER $\beta$  also bound the 342–646 fragment, which contained only NR box i (Fig. 1C). These interactions were of approximately equal intensity. There was also a weak, ligand-independent interaction with GST-RAC3 1–407. Identical results were obtained for ER $\alpha$  (data not shown). A Coomassie Blue-stained polyacrylamide gel of the GST-RAC3 fusion protein confirmed the identity of each GST-RAC3 fusion protein and approximately equal protein concentrations in each lane (data not shown). Thus, the VDR and ER $\beta$  display different binding patterns for RAC3 fragments, with the VDR interacting preferentially with regions containing NR box iii and the ER $\beta$  interacting equally well with regions containing any of the three NR boxes and the N-terminal basic helix-loop-helix-PAS domain.

**NR Box Peptides Differentially Compete with Nuclear Receptors for RAC3 Binding**—We then wanted to investigate the relative importance of individual NR boxes within the RAC3-RID in mediating the interactions between RAC3 and the VDR or ER $\beta$ . Peptides were synthesized corresponding to NR boxes i, ii, and iii, which were incubated with  $^{35}$ S-labeled receptor and 1  $\mu$ M ligand before probing the GST-RAC3 613–752 fragment in the Far Western assay. With the VDR, peptides corresponding to the second and third LXXLL motif were able to compete away the RAC3-RID interaction with VDR in a dose-dependent manner (Fig. 2A). Upon quantifying the data, it was evident that peptide iii was a more potent inhibitor than peptide ii, whereas the peptide comprising NR box i had little, if any, effect on the VDR-RID interaction (Fig. 2B). A control experiment demonstrated that the effects of these peptides were specific, for a random peptide did not alter the interaction between the  $^{35}$ S-VDR and GST-RAC3-RID (Fig. 2C). We again identified a different pattern when the same experiment was done with the ER $\beta$  (Fig. 2D). Here, all three peptides were able to compete efficiently for ER $\beta$  binding with the RAC3-RID, with peptide ii being the most potent (Fig. 2E). Thus, these data reveal receptor-specific preferences for interactions between RAC3 and different nuclear receptors, for the VDR and ER $\beta$  have different affinities for the NR boxes of RAC3.

**NR Box Mutations Can Impair RAC3 Interactions with Nuclear Receptors *In Vitro***—To assess the integrity of the LXXLL motif in mediating the interaction between the RAC3-RID and nuclear receptors, we used site-directed mutagenesis to switch the leucine residues of each motif to alanines (Fig. 3A). The mutants were made using the GST-RAC3-RID fusion as the template and tested for their ability to interact with the VDR or ER $\beta$  in GST pull-down assays. The wild-type RAC3-RID was able to pull down a significant amount of  $^{35}$ S-VDR in the presence of 1  $\mu$ M vitamin D (Fig. 3B). This interaction was specific, for GST alone pulled down much less  $^{35}$ S-VDR. Mutations in NR boxes i or ii displayed wild-type binding. However, when NR box iii was mutated, the RID-VDR interaction was greatly reduced to a level only slightly higher than background binding to GST alone. GST-RAC3 342–646, which contained only NR box i, also had minimal binding to the VDR, consistent

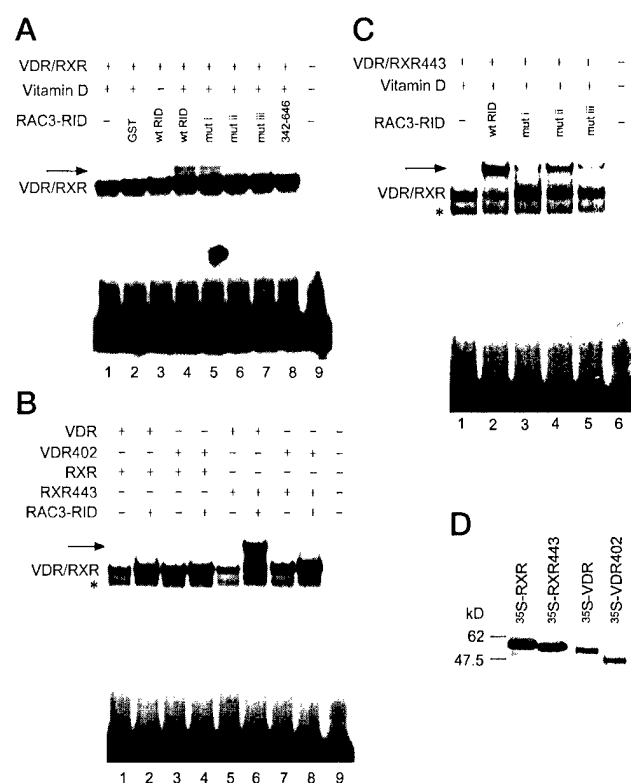


**FIG. 2. Peptides corresponding to the NR boxes of the RAC3-RID can compete for VDR and ER $\beta$  binding with the RAC3-RID.** A, Far Western assay using  $^{35}$ S-VDR to probe GST-RAC3-RID in the presence of 1  $\mu$ M vitamin D and given concentration of each peptide. B, the data from A was quantified by PhosphorImager and plotted as percent GST-RAC3-RID binding to  $^{35}$ S-VDR versus peptide concentration. 100% RID binding represents the density of the band in the absence of peptide.  $\diamond$ , peptide i;  $\blacksquare$ , peptide ii;  $\blacktriangle$ , peptide iii. C, Far Western assay demonstrating that inhibition of  $^{35}$ S-VDR interaction with GST-RAC3-RID by peptide iii is specific. Peptide iii abolishes nearly all the interaction, whereas a control, random peptide has no effect. D, Far Western assay using  $^{35}$ S-ER $\beta$  to probe GST-RAC3-RID in the presence of 1  $\mu$ M estradiol and given concentration of each peptide. E, the data from D was quantified by PhosphorImager and plotted as the percent GST-RAC3-RID binding to  $^{35}$ S-ER $\beta$  versus peptide concentration.  $\diamond$ , peptide i;  $\blacksquare$ , peptide ii;  $\blacktriangle$ , peptide iii.

with the Far Western assay (Fig. 1B). Equal protein concentrations of each GST fusion confirmed the specificity of these findings. Thus, these data support the above observations in implicating NR box iii as being most critical to RAC3 interaction with the VDR.

The wild-type RID was also able to pull down significant amounts of  $^{35}$ S-ER $\beta$  in the GST pull-down assay (Fig. 3C). In contrast to the VDR, an alanine substitution for leucine in any of the three NR boxes weakened the interaction of the RAC3-RID with ER $\beta$ , with the mutation of NR box ii being the most deleterious, again supporting the results of LXXLL peptide competition experiments (Fig. 2B). However, with each mutation, significant binding above background between the RAC3-RID and  $^{35}$ S-ER $\beta$  was still observed. Furthermore, the GST-RAC3 342–646 fragment, with only NR box i, was able to interact efficiently with ER $\beta$  (Fig. 3C) as in the Far Western assay (Fig. 1C). These data suggest that although all three motifs are capable of interacting with ER $\beta$  separately, none of them is absolutely required for the interaction. In contrast, NR box iii of RAC3 appears to be essential for the interaction with the VDR.

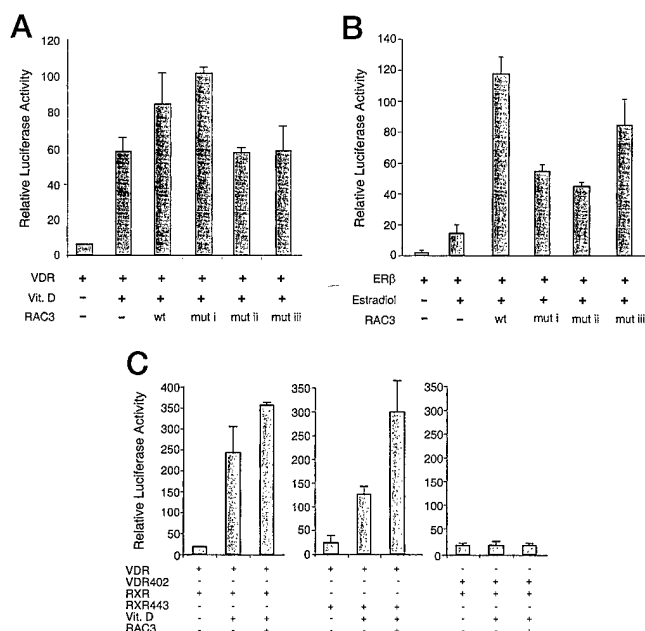
**RAC3-RID Interactions with DNA-bound Nuclear Receptors**—The above data provide compelling evidence that the VDR interacts specifically with RAC3 in solution via the LXXLL motifs of the RAC3 RID, particularly NR box iii. To gain further insight into the function of NR boxes in coactivator-VDR interactions on a heterodimeric complex bound to DNA, we performed gel-shift assays with VDR/RXR heterodimers on a DR3 element in the presence of wild-type or mutant RAC3-RID (Fig. 4). The VDR/RXR heterodimer bound strongly to the  $^{32}$ P-labeled DR3 probe and was unaffected by the addition of GST alone (Fig. 4A, lanes 1 and 2). The addition of the RAC3-RID resulted in a ligand-dependent shift of the



**FIG. 4. Mutation of RAC3 NR boxes inhibits RAC3-RID binding to DNA-bound VDR/RXR heterodimer.** *A*, gel-shift assay of the effects of NR box mutations (*mut*) on RAC3-RID binding to DR3-bound VDR/RXR. 1.5  $\mu$ l of each  $^{35}$ S-labeled nuclear receptor was added to a binding reaction (see "Experimental Procedures") containing 1  $\mu$ M vitamin D, equal amounts of the indicated GST fusion protein, and the [ $^{32}$ P]dCTP-labeled DR3 probe. The arrow indicates the RID-receptor complex. *wt*, wild type. *B*, the RXR AF-2 domain can interfere with RID binding to VDR/RXR, whereas the VDR AF-2 domain is required for the interaction. The gel-shift assay was performed as in *A*, except the AF-2-truncated RXR443 or VDR402 was used where indicated. \*, non-specific band from lysate. *C*, the VDR/RXR443 heterodimer has different NR box preferences than the wild-type receptor heterodimer. The gel-shift assay was performed as in *A*. \*, nonspecific band from lysate. *D*, autoradiograph confirming the equal expression of the  $^{35}$ S-labeled receptors used in *B* and *C*.

We next analyzed the involvement of nuclear receptor AF-2 domains in regulating the interaction between the RAC3-RID and the VDR/RXR heterodimer (Fig. 4B). Using the gel-shift assay, we compared the ability of the RID to bind the wild-type heterodimer *versus* the VDR/RXR443 and VDR402/RXR heterodimers, in which the AF-2 domain of RXR or VDR had been deleted, respectively. As demonstrated above, the RID was able to bind the DNA-bound, wild-type VDR/RXR complex (Fig. 4B, *lane 2*). Deletion of the VDR AF-2 domain did not affect the formation of the heterodimer-DNA complex but resulted in the loss of the RID-shifted complex (*lane 4*). This suggests that the VDR AF-2 domain is required for interaction of RAC3 with the heterodimer and that RXR AF-2 domain alone is not sufficient for the interaction. Interestingly, deletion of the RXR AF-2 domain resulted in a much stronger shift of the heterodimeric complex by the RAC3-RID (*lane 6*) without affecting heterodimer formation (*lane 5*), suggesting that the RXR AF-2 domain can inhibit the interaction between RAC3 and VDR/RXR. The strong interaction was abolished upon deletion of the VDR AF-2 domain (*lane 8*), further supporting a requirement of VDR AF-2 helix 12 for RAC3 binding to the DNA-bound heterodimer.

**Effects of NR Box Mutations on RAC3 Coactivation Function *In Vivo***—RAC3 has previously been shown to enhance the transcriptional activity of the RAR and progesterone receptor (37). However, its effect on VDR and ER $\beta$  function *in vivo* has not been demonstrated. To address this, we performed transient transfection assays in HEK293 and CV-1 cells using luciferase reporters harboring either two copies of the vitamin D response element of the osteopontin gene for VDR studies or a consensus ERE element for ER $\beta$  studies (Fig. 5). Transfection of the VDR into CV-1 cells minimally activated the vitamin D response element driven reporter (Fig. 5A). However, treating



**FIG. 5. Mutation of RAC3 NR boxes blocks coactivation of VDR and ERβ activity *in vivo*.** A, mutation (*mut*) of NR box ii or iii inhibits RAC3 enhancement of VDR activity. CV-1 cells were transfected with expression plasmids for VDR, wild-type (*wt*), or mutant RAC3 and the Spx2-luciferase reporter and treated with 40 nM vitamin D for 24 h where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β-galactosidase activity. B, mutation of NR box i, ii, or iii inhibits RAC3 enhancement of ERβ activity. CV-1 cells were transfected with expression plasmids for ERβ, wild-type, or mutant RAC3 and the ERE-luciferase reporter and treated with 10 nM estradiol for 24 h where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β-galactosidase activity. C, deletion of the RXR AF-2 domain results in enhanced coactivation of VDR/RXR activity by RAC3. HEK293 cells were transfected with the appropriate nuclear receptor expression plasmids along with expression plasmids for RAC3 and Spx2-luciferase and treated with 10 nM vitamin D where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β-galactosidase activity.

these cells with vitamin D strongly stimulated its activity. Cotransfection of RAC3 further enhanced VDR transcriptional activation by approximately 50%, consistent with the coactivation function of RAC3 (37).

We then analyzed the role of the NR boxes in mediating the ability of RAC3 to potentiate VDR activity. Mutations of each NR box of the RAC3-RID were made in the context of the full-length RAC3 protein and tested for their ability to coactivate the VDR in transient transfection assays (Fig. 5A). Mutation of NR box i did not inhibit RAC3 enhancement of VDR transactivation, consistent with its inability to block the interaction of RAC3 with VDR *in vitro*. However, the RAC3- NR box ii or iii mutations reduced the function of RAC3 in enhancing VDR activity (Fig. 5A), consistent with their requirement for RAC3 binding to the DNA-bound VDR/RXR heterodimer in gel-shift assays (Fig. 4A). Taken together, it is clear that NR boxes ii and iii are both involved in RAC3 regulation of VDR function, whereas the role of NR box i appears minimal.

We then repeated these experiments with the ERβ (Fig. 5B). Estradiol treatment of CV-1 cells transfected with the ERβ and the ERE-luciferase reporter activated reporter expression approximately 8-fold. Cotransfection of wild-type RAC3 resulted in a strong enhancement of ERβ activity. It is evident from these data that RAC3 is a more potent coactivator for the ERβ than for the VDR. Cotransfection of RAC3 expression plasmids containing mutations in NR boxes i, ii, or iii all suppressed the ability of RAC3 to coactivate the ERβ, with the NR box ii mutant having the greatest and NR box iii mutant having more

modest effects on RAC3 function. Thus, these *in vivo* data correlate with the *in vitro* data in implicating all three NR boxes of the RAC3-RID as being important for RAC3 regulation of ERβ function.

Finally, we decided to assess the functional consequences of the antagonism of RAC3 interaction with DNA-bound VDR/RXR by the RXR AF-2 domain observed in gel-shift assays (Fig. 4B). To do this, we compared the ability of RAC3 to coactivate VDR/RXR and VDR/RXR443 activities by transient transfection assays (Fig. 5C). When wild-type VDR and RXR were expressed in HEK293 cells, RAC3 was able to enhance transcriptional activation by approximately 50% (Fig. 5C, left). However, when VDR was coexpressed with RXR443, RAC3 displayed a 2.5-fold enhancement of receptor activity (Fig. 5C, center). As expected, the VDR402 mutant was transcriptionally inactive, and RAC3 could not modulate its activity (Fig. 5C, right). Thus, these *in vivo* data are consistent with the gel-shift data in demonstrating that the RXR AF-2 domain can inhibit RAC3 modulation of the VDR/RXR heterodimer, whereas the VDR AF-2 domain is absolutely required for this regulation.

#### DISCUSSION

In this study, we have investigated the role of the NR boxes of RAC3 in mediating the ability of this coactivator to bind and coactivate the VDR and ERβ. We found that NR box iii is most critical to VDR binding, whereas NR boxes i, ii, and iii are involved in ERβ interaction. Peptides corresponding to these respective motifs were able to compete with the RAC3-RID for VDR and ERβ binding. The integrity of the motifs themselves was also important, for mutations in specific NR boxes inhibited RAC3 interaction with these receptors in solution and when bound to DNA. The AF-2 domain of VDR is required for binding of the RAC3-RID to DNA-bound VDR/RXR, whereas the AF-2 domain of RXR was able to antagonize this interaction. Removal of this inhibitory AF-2 helix of RXR enhances ligand-dependent binding of RAC3 to the VDR/RXR443 heterodimer and alters the NR box requirements. Furthermore, the mutation of NR box ii or iii blocked the ability of RAC3 to enhance transcriptional activation by the VDR *in vivo*. In contrast, mutation of NR boxes i, ii, or iii reduced RAC3 coactivation of ERβ. Together, these *in vitro* and *in vivo* studies suggest a mechanistic difference in the manner by which RAC3 regulates VDR and ERβ activities.

The NR boxes are highly conserved among the SRC family of coactivators (18). Our data and that of others clearly reveal that multiple motifs are necessary for high affinity interactions with nuclear receptors (23, 32, 33). With the DNA-bound VDR/RXR heterodimer, we found that mutation of NR boxes ii or iii of RAC3 weakens the interaction with the RAC3-RID. In contrast, NR box iii of RAC3 is the only critical motif for interaction with VDR in solution. Therefore, it is likely that each motif binds to each monomer of the receptor heterodimer, consistent with the structure of a peroxisome proliferator-activated receptor γ-LBD dimer co-crystallized with a fragment of SRC1 containing two NR boxes (12). Our data on ERβ suggest that all three NR boxes of the RAC3-RID are involved for a wild-type interaction, whereas the presence of two motifs is sufficient for a strong interaction, motif ii being most important. In light of this finding, RAC3 may utilize motif ii in combination with motif i or iii for efficient interaction with the ERβ homodimer. The integrity of the other motif may be critical to the overall conformation of the coactivator or potentially make an additional contact with another region of the receptor. Support for the latter possibility can be found in recent studies detailing the enhancement of the N-terminal AF-1 activation function of nuclear receptors by SRC coactivators (38, 39). The presence of multiple NR boxes also likely provides coactivators the flexi-

bility to interact with a broad range of nuclear receptors, resulting in the different preferences that are observed between nuclear receptors and distinct motifs, depending on the precise structural nuances of each receptor-coactivator interface. This is evident upon comparing the NR box requirements of the VDR/RXR heterodimer *versus* those of VDR/RXR443. Deletion of the AF-2 helix of RXR not only enhances RAC3-RID binding to the heterodimer but also switches the NR box preferences from motifs ii/iii to motifs i/iii. Finally, amino acids flanking the NR boxes also likely contribute to the specificity of interaction (15, 32), for, despite the high homology between the RAC3 NR boxes, peptides comprising each motif and surrounding residues displayed different affinities for VDR or ER $\beta$  binding. Thus, it is clear that the multiple NR boxes do not serve merely redundant functions.

Our finding that the AF-2 domain of RXR can interfere with RAC3-RID binding to a DNA-bound VDR/RXR heterodimer is consistent with studies suggesting allosteric inhibition of coactivator binding to RAR/RXR by the RXR AF-2 domain (36). This inhibition may be the result of competition between the AF-2 domain of RXR and the LXXLL motif for the coactivator binding site on the other receptor (11, 12, 36). In the antagonist-bound ER $\alpha$ -LBD crystal structure, the AF-2 domain occupies the coactivator binding groove, mimicking the hydrophobic interactions of the NR box peptide with this domain in the agonist-NR box peptide-receptor complex (11). Biochemical studies with RAR/RXR and SRC1 support these observations, for binding of RAR- and RXR-specific ligands enhance SRC1 interaction with the receptor dimer relative to the interaction in the presence of either ligand alone (36). Presumably, one ligand binding recruits a single NR box to the receptor dimer, which displaces the AF-2 domain from the coactivator binding site and relieves allosteric inhibition, allowing the second ligand to bind the other receptor monomer. This, in turn, enhances the interaction with coactivator by recruiting a second NR box (36). In the case of wild-type receptors, hormone does stimulate RAC3-RID binding to the heterodimer, but only weakly compared with the VDR/RXR443 dimer, where a very strong, vitamin D-dependent interaction is observed. These observations are confirmed by *in vivo* studies demonstrating that RAC3 can coactivate VDR/RXR443 activity to a greater extent than VDR/RXR activity. Hormone binding and RID recruitment must not be able to displace every RXR AF-2 domain from the coactivator binding site of the partnering receptor; thus, fewer RID molecules are able to bind in the presence of the RXR AF-2 domain. This suggests that the AF-2 domain of RXR plays a critical role in regulating RAC3 modulation of receptor function. However, other possibilities may explain this finding, foremost being the hypothesis that deletion of the AF-2 domain of RXR results in a conformational change of the VDR/RXR443 dimer that enhances its affinity for the RAC3-RID.

Our data demonstrate for the first time that RAC3 can enhance the transcriptional activation function of the VDR and ER $\beta$  and that this coactivation activity depends on different NR box requirements. Several other cofactors have been found to stimulate VDR activity, including SRC1, GRIP1/TIF2, NCoA-62, and the DRIP (VDR-interacting proteins) complex (13, 40–42), whereas SRC1 can coactivate the ER $\beta$  (43). The role of multiple coactivators in the function of the VDR *in vivo* is unknown, but several possibilities exist that suggest that the function of these coactivators is not completely redundant. First, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3 is expressed at a high level in placenta, heart, and HeLa cells relative to TIF2 and SRC1 (20); thus, it may serve a more prominent role in receptor function in these cells. Second, dif-

ferent coactivators may serve different functions that in total result in maximal transcriptional activation by the VDR. For example, RAC3 can interact with CBP; thus, RAC3 may recruit CBP to the VDR. SRC1 has intrinsic histone acetylation activity, and the DRIP VDR-interacting proteins complex may remodel nucleosomes (21, 44), which also may contribute to the overall function of the VDR in stimulating target gene expression. Finally, we cannot rule out the existence of a complex containing multiple coactivators, which synergize to potentiate VDR activity.

In summary, our data establish RAC3 as a potent coactivator of the vitamin D receptor and estrogen receptor  $\beta$ . Interestingly, RAC3 modulates the function of these receptors differently via interactions that depend on specific LXXLL motifs in the RAC3 receptor-interacting domain. Although the biological role of RAC3 in nuclear receptor function remains to be explored, this study sheds light on the molecular mechanisms of RAC3 regulation of receptors that will hopefully lead to a better understanding of SRC coactivator function *in vivo*.

**Acknowledgments**—We thank Dr. Jan-Ake Gustafsson and Dr. Ming-Jer Tsai for the ER $\beta$  expression plasmid and ERE-luciferase reporter, respectively. We also thank Dr. Robert Carraway at the University of Massachusetts Medical School peptide core facility for peptide synthesis and Mausumi Bhaumik for technical assistance.

#### REFERENCES

- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83**, 835–839
- Chen, J. D., and Evans, R. M. (1995) *Nature* **377**, 454–457
- Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) *Nature* **377**, 397–404
- Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) *Cell* **89**, 373–380
- Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 43–48
- Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePamphilis, R. A. (1997) *Nature* **387**, 49–55
- Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753–758
- Wagner, R. L., Apriletti, J. W., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995) *Nature* **378**, 690–697
- Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **378**, 681–689
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**, 377–382
- Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**, 927–937
- Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395**, 137–143
- Kraichely, D. M., Collins, J. J., III, DeLisle, R. K., and MacDonald, P. N. (1999) *J. Biol. Chem.* **274**, 14352–14358
- Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12**, 3343–3356
- Mak, H. Y., Hoare, S., Henttu, P. M., and Parker, M. G. (1999) *Mol. Cell. Biol.* **19**, 3895–3903
- Feng, W., Ribeiro, R. C., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) *Science* **280**, 1747–1749
- McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) *Endocr. Rev.* **20**, 321–344
- Chen, J. D., and Li, H. (1998) *Crit. Rev. Eukaryotic Gene Expression* **8**, 169–190
- Blanco, J. C., Minucci, S., Lu, J., Yang, X. J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) *Genes Dev.* **12**, 1638–1651
- Li, H., and Chen, J. D. (1998) *J. Biol. Chem.* **273**, 5948–5954
- Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389**, 194–198
- Yao, T. P., Ku, G., Zhou, N., Scully, R., and Livingston, D. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10626–10631
- Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) *EMBO J.* **17**, 507–519
- Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 677–684
- Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996) *Nature* **383**, 99–103
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C.,

- Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85**, 403-414
27. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) *Nature* **382**, 319-324
  28. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**, 569-580
  29. Bannister, A. J., and Kouzarides, T. (1996) *Nature* **384**, 641-643
  30. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87**, 953-959
  31. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387**, 733-736
  32. McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) *Genes Dev.* **12**, 3357-3368
  33. Leers, J., Treuter, E., and Gustafsson, J. A. (1998) *Mol. Cell. Biol.* **18**, 6001-6013
  34. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998) *Mol. Endocrinol.* **12**, 302-313
  35. Chen, J. D., Umesono, K., and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7567-7571
  36. Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998) *Nature* **395**, 199-202
  37. Li, H., Gomes, P. J., and Chen, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8479-8484
  38. Ma, H., Hong, H., Huang, S. M., Irvine, R. A., Webb, P., Kushner, P. J., Coetzee, G. A., and Stallcup, M. R. (1999) *Mol. Cell. Biol.* **19**, 6164-6173
  39. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McInerney, E., Katzenellenbogen, B. S., Stallcup, M. R., and Kushner, P. J. (1998) *Mol. Endocrinol.* **12**, 1605-1618
  40. Takeyama, K., Masuhiro, Y., Fuse, H., Endoh, H., Murayama, A., Kitanaka, S., Suzawa, M., Yanagisawa, J., and Kato, S. (1999) *Mol. Cell. Biol.* **19**, 1049-1055
  41. Rachez, C., Suldan, Z., Ward, J., Chang, C. P., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998) *Genes Dev.* **12**, 1787-1800
  42. Baudino, T. A., Kraichely, D. M., Jefcoat, S. C., Jr., Winchester, S. K., Partridge, N. C., and MacDonald, P. N. (1998) *J. Biol. Chem.* **273**, 16434-16441
  43. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. (1997) *Mol. Endocrinol.* **11**, 353-365
  44. Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) *Nature* **398**, 824-828



## Sequestration and Inhibition of Daxx-Mediated Transcriptional Repression by PML

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Received 19 August 1999/Returned for modification 24 September 1999/Accepted 23 November 1999

**PML fuses with retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) in the t(15;17) translocation that causes acute promyelocytic leukemia (APL). In addition to localizing diffusely throughout the nucleoplasm, PML mainly resides in discrete nuclear structures known as PML oncogenic domains (PODs), which are disrupted in APL and spinocellular ataxia cells. We isolated the Fas-binding protein Daxx as a PML-interacting protein in a yeast two-hybrid screen. Biochemical and immunofluorescence analyses reveal that Daxx is a nuclear protein that interacts and colocalizes with PML in the PODs. Reporter gene assay shows that Daxx drastically represses basal transcription, likely by recruiting histone deacetylases. PML, but not its oncogenic fusion PML-RAR $\alpha$ , inhibits the repressor function of Daxx. In addition, SUMO-1 modification of PML is required for sequestration of Daxx to the PODs and for efficient inhibition of Daxx-mediated transcriptional repression. Consistently, Daxx is found at condensed chromatin in cells that lack PML. These data suggest that Daxx is a novel nuclear protein bearing transcriptional repressor activity that may be regulated by interaction with PML.**

Acute promyelocytic leukemia (APL) arises as a result of chromosomal translocation involving the retinoic acid (RA) receptor  $\alpha$  (RAR $\alpha$ ) gene on chromosome 17 fused with either the promyelocytic leukemia gene (PML) on chromosome 15, the promyelocytic leukemia zinc finger gene (PLZF) on chromosome 11, the nucleophosmin/B23 (NPM) gene on chromosome 5, or the nuclear mitotic apparatus gene (NuMA) on chromosome 11 (30, 39). The t(15;17) translocation between PML and RAR $\alpha$  accounts for nearly all APL cases. This translocation creates an oncogenic fusion protein, PML-RAR $\alpha$ , which contains both the DNA-binding domain (DBD) and ligand-binding domains of RAR $\alpha$  and the N terminus of PML. Transgenic mice that overexpress PML-RAR $\alpha$  or PLZF-RAR $\alpha$  developed an APL-like phenotype (9, 21, 26), suggesting that these fusion proteins are directly involved in APL pathogenesis. Recent studies have focused on analyzing the functional properties of PML-RAR $\alpha$  and PLZF-RAR $\alpha$  (20, 22, 25, 40) in order to understand the molecular basis of leukemogenesis. Both fusion proteins form homodimers that bind to RA response elements and interact with the nuclear receptor corepressors SMRT (silencing mediator for retinoid and thyroid hormone action) and N-CoR (nuclear receptor corepressor), which in turn recruit a histone deacetylase complex (1, 27, 40, 46). Pharmacological concentrations of all-*trans*-RA (atRA) induce dissociation of the corepressors from PML-RAR $\alpha$ , but not PLZF-RAR $\alpha$ , due to the presence of an additional, RA-insensitive corepressor-interacting surface on PLZF. This differential degree of dissociation of corepressors induced by atRA correlates with the ability of histone deacetylase inhibitors and atRA to induce terminal differentiation of these two subtypes of APL cells. These findings indicate that abnormalities in transcriptional repression by the oncogenic fusion proteins may be involved in leukemogenesis.

PML belongs to a family of proteins characterized by the presence of a RING finger domain (8). RING finger proteins are implicated in transcriptional regulation, and some members of the RING family are associated directly with chromatin (53). Ablation and overexpression experiments suggest an important role of PML in the regulation of cell growth, hematopoietic cell differentiation, tumorigenesis, apoptosis, and RA signaling (44, 63). In normal cells, PML is concentrated within 10 to 20 nuclear structures known as nuclear domains 10 (ND10), Krüppel bodies, nuclear bodies, or PML-oncogenic domains (PODs) (2, 17, 33, 59, 65). The POD structure is disrupted in the t(15;17) translocated APL cells (17, 33, 65), presumably through interaction of wild-type PML with PML-RAR $\alpha$ . Interestingly, the POD structure reorganizes upon treatment with atRA or arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), a process that correlates with differentiation of APL cells, indicating that the POD structure might affect promyelocyte differentiation.

In addition to PML, the POD contains several other proteins, including the 100-kDa nuclear protein antigen (Sp100) (2), the small ubiquitin-related modifier (SUMO-1 [41], also known as PML-interacting clone 1 [PIC1] [7], ubiquitin-like 1 [UBL1] [57], or sentrin [48]), and the 140-kDa protein (Sp140) (6). Sp100 is a nuclear antigen recognized by autoantibodies from patients with primary biliary cirrhosis (62). Expression of both PML and Sp100 are upregulated by interferon (23). SUMO-1 was recently identified as a ubiquitin-like protein that forms covalent conjugates with PML and Sp100 (7, 58). In addition, the CREB-binding protein (CBP) and the retinoblastoma tumor suppressor (pRB) have been found in the PODs (35, 61). Also, the PODs are targets of several viral proteins, which alter POD structure (11, 14, 18). Although there is evidence for POD's role in transcriptional activation (15, 35), DNA replication (19), apoptosis (51, 64), and viral infection (14, 42), the precise function of PODs in these processes remains unclear.

We have sought to understand the function of PODs through identification of PML-interacting proteins that also localize in the PODs. By using the yeast two-hybrid system, we identified SUMO-1 and the Fas-binding protein Daxx (68)

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(J. D. Chen and R. M. Evans, unpublished data). Daxx has been shown to promote Fas-mediated apoptosis through activation of the Jun NH<sub>2</sub>-terminal kinase (JNK) and JNK kinase kinase ASK1 (apoptosis signal-regulating kinase 1) (12). Recent data suggest that Daxx is not sufficient for Fas-mediated apoptosis, since a Fas mutant that selectively binds to Daxx but not the Fas-adaptor death domain-containing protein (FADD/MORT1) failed to induce apoptosis (13). Other evidence suggests that Daxx may interact with the centromeric protein- $\alpha$  (CENP-C) and may bind to a steroidogenic factor 1 (SF-1)-like DNA element (32, 50). Therefore, the exact mechanism by which Daxx regulates Fas-mediated apoptosis may involve nuclear processes.

In the present study, we have characterized both biochemical and functional interactions between Daxx and PML. Daxx resides primarily in the cell nucleus, where it forms a complex with PML. Confocal immunofluorescence data demonstrate that Daxx colocalizes with PML in the PODs, and such colocalization persists in NB4 APL cells (36) before and after treatment with atRA and As<sub>2</sub>O<sub>3</sub>. Daxx possesses strong transcriptional repressor activity and appears to interact directly with histone deacetylases. Intriguingly, overexpression of PML inhibits Daxx-mediated transcriptional repression and, in cells that lack PML, Daxx is preferentially associated with condensed chromatin. Our data reveal a new role for Daxx in transcriptional repression and suggest a novel function of PML and the POD structure in the suppression of transcriptional repression.

#### MATERIALS AND METHODS

**Yeast two-hybrid system.** The screening of PML-interacting proteins was conducted by the yeast two-hybrid system by using the Y190 strain as previously described (16). The Gal4 DBD (amino acids 1 to 147) fusion of full-length PML (29) was constructed in the yeast vector pAS1 (16). The resulting Gal4 DBD-PML fusion protein was used as bait to screen a Gal4 activation domain (AD)-fused human B-lymphocyte cDNA library in the pACT expression vector (16). About 10<sup>6</sup> yeast transformants were screened on selection plates containing 50 mM 3-aminotriazole (Sigma). For ligand treatment, the culture was incubated in the presence of ligand or solvent (control) for 24 h before measuring the  $\beta$ -galactosidase ( $\beta$ -Gal) activity.

**Biochemical cell fractionation.** HeLa cells ( $2 \times 10^6$ ) were harvested into 500  $\mu$ l of CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>–5 mM EDTA–1 mM phenylmethylsulfonylfluoride–proteinase inhibitors). Cells were allowed to swell for 5 min on ice, Dounce homogenized 35 times, and centrifuged at 7,500 rpm for 5 min to pellet nuclei and debris. The supernatant (cytosol plus plasma membrane) was then spun at 25,000 rpm for 30 min to pellet the membrane. The nucleus-debris pellet was resuspended in 1 ml of TSE buffer (10 mM Tris, pH 7.5; 300 mM sucrose; 1 mM EDTA) and Dounce homogenized 30 times, followed by centrifugation at 5,000 rpm for 5 min. The pellet was resuspended and washed twice to obtain the final nucleus pellet. Equal amounts of protein in each fraction were analyzed by Western blotting.

**Western blotting.** Western blotting was conducted by using the enhanced chemiluminescence reagents according to the manufacturers' recommendation (Amersham). The affinity purified anti-Daxx polyclonal antibodies were raised against glutathione *S*-transferase (GST)-Daxx (amino acids 556 to 740) fusion protein and subsequently purified with the GST-Daxx protein column as described earlier (24). Anti-Gal4-DBD antibody was purchased from Santa Cruz, and anti-HDAC1 antibody was from Upstate Biotechnology.

**Co-IP.** Coimmunoprecipitation (Co-IP) was conducted according to a standard procedure by using the protein A-agarose beads (Santa Cruz) (24). Nuclear extracts were prepared as described earlier (3). HeLa and NB4 cells were lysed in cell lysis buffer (0.4 M NaCl, 0.2 mM EGTA, 10% glycerol, 1% NP-40), and cell extracts were precleared by incubating them with protein-A agarose beads for 1 h at room temperature. The affinity-purified IP antibodies were conjugated with protein A-agarose beads in cell lysis buffer for 2 h at room temperature. The antibody-protein A-agarose was collected by brief centrifugation and incubated with cell extracts (100  $\mu$ g) overnight at 4°C. The precipitates were collected by centrifugation and washed five times with excess phosphate-buffered saline containing 0.1% NP-40. The final precipitate was dissolved in sodium dodecyl sulfate A (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

**Immunofluorescence and confocal microscopy.** Cells were grown on cover glasses (VWR Scientific), fixed in a methanol-acetic acid (1:1) mixture on dry ice

for 2 min and processed for immunofluorescence staining as described elsewhere (17). For NB4 cells, the cover glasses were coated with poly-L-lysine before seeding the cells. After immunostaining, cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate) (Sigma). Confocal microscopy was conducted with a Leica TCS SP spectral laser scanning confocal microscope. Channel cross-talk was avoided by reducing the intensity of the excitation laser beam in the absence of the other excitation laser. Standard epifluorescence microscopy was performed on an Olympus IX-70 microscope equipped with a back-illuminated cool charge-coupled device (CCD) camera (Princeton Instruments), and the image was processed by using the MetaMorph software (Universal Imaging Corp.).

**Transient-transfection assay.** Transient transfection was conducted using a standard calcium phosphate precipitate method as described earlier (3). Cultured cells were maintained in Dulbecco modified Eagle medium or RPMI medium (for NB4 cells) supplemented with 10% fetal bovine serum (Gibco). Twelve hours prior to transfection,  $2 \times 10^4$  cells were plated in each well of 12-well plates. Transfected cells were refed with fresh media and harvested 36 to 48 h after transfection. Transfected cells in each well were lysed and processed for luciferase and  $\beta$ -Gal assay as described elsewhere (38). The luciferase activity was determined with an MLX plate luminometer (Dynex) and normalized with the cotransfected  $\beta$ -Gal.

**Far-Western blot.** GST fusion proteins were expressed in DH5 $\alpha$  cells and purified by standard glutathione agarose beads according to manufacturer's recommendation (Pharmacia). The purified proteins were separated by SDS-PAGE and electroblotted onto a nitrocellulose filter in transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 0.01% SDS). Proteins were denatured with 6 M guanidine hydrochloride (GnHCl) and renatured by stepwise dilution of GnHCl. Filters were blocked and hybridized overnight with <sup>35</sup>S-labeled protein as described elsewhere (38). The membrane was then washed three times with hybridization buffer, and the bound probe was detected by autoradiography.

**GST pull-down assay.** The GST pull-down assay was conducted according to a protocol as described earlier (24). Briefly, 5  $\mu$ g of glutathione agarose-protein beads was incubated with 5  $\mu$ l of in vitro-translated <sup>35</sup>S-labeled protein with moderate shaking at 4°C overnight in binding buffer (20 mM HEPES, pH 7.7; 75 mM KCl; 0.1 mM EDTA; 2.5 mM MgCl<sub>2</sub>; 0.05% NP-40; 1 mM dithiothreitol; 1 mg of bovine serum albumin per ml). The bound protein was washed three times with the binding buffer, and the beads were collected by centrifugation. The bound protein was eluted in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

**Site-directed mutagenesis.** Site-directed mutagenesis was conducted by using the Quick-Change site-directed mutagenesis kit according to manufacturer's instruction (Stratagene). A mammalian hemagglutinin (HA)-PML vector was used as a template, and mutagenesis was conducted in three rounds consecutively on the same template. The mutated construct was confirmed by DNA sequencing by using dideoxynucleotide chain-termination reactions and Sequenase (U.S. Biochemicals).

#### RESULTS

**Identification of Daxx as a PML-interacting protein.** In the yeast two-hybrid screen, we identified a PML-interacting clone that encodes the C-terminal 184 amino acids of Daxx (32, 50). Yeast two-hybrid assay shows that this Daxx clone interacts with Gal4 DBD fusions of both PML and PML-RAR $\alpha$  but not SP100 (Fig. 1A), suggesting that Daxx may be a PML-interacting protein. Since atRA binds to PML-RAR $\alpha$  in a way similar to that of wild-type RAR $\alpha$  (4), we determined the effect of atRA on interaction between Daxx and PML-RAR $\alpha$  (Fig. 1B). atRA inhibits the two-hybrid interaction between Daxx and PML-RAR $\alpha$  efficiently and in a dose-dependent manner. The inhibition of binding is slightly more sensitive with the long form of PML-RAR $\alpha$  than with the short form, a finding consistent with the higher affinity of the long form of PML-RAR $\alpha$  for atRA (4). This atRA-dependent inhibition of binding is specific, for atRA has no effect on the interaction between PML and Daxx while it enhances the interaction between PML-RAR $\alpha$  and the coactivator RAC3 (38). Also, the thyroid hormone triiodothyronine that does not bind PML-RAR $\alpha$  also has no effect on the interaction between Daxx and PML-RAR $\alpha$ . These data suggest that Daxx is a PML-interacting protein that may also associate with the oncoprotein PML-RAR $\alpha$  in the absence of atRA.

**Daxx forms a complex with PML in vivo.** In addition to being diffusely distributed in the cytoplasm, PML is mainly a nuclear protein, while Fas is a transmembrane cell surface

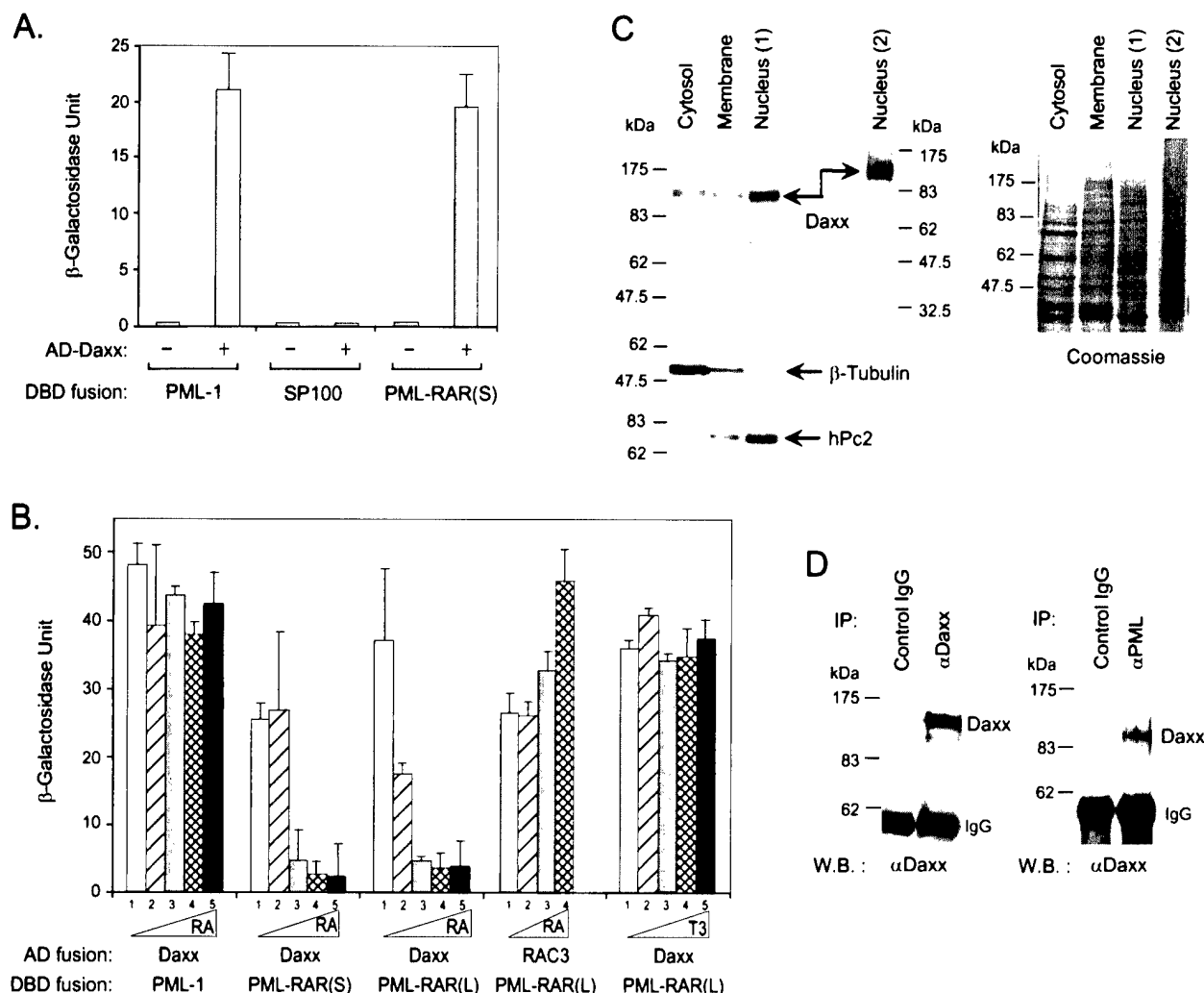


FIG. 1. Interaction between Daxx and PML in vivo. (A) Interaction of Daxx with PML in yeast two-hybrid system. The average  $\beta$ -Gal activities of three transformants expressing the indicated combinations of Gal4 AD and DBD fusion proteins were determined as described in Materials and Methods. The AD-Daxx fusion protein contains amino acids 556 to 740 of human Daxx. The DBD fusion proteins contain full-length PML-1, SP100, and PML-RAR $\alpha$  short form, respectively. The minus sign indicates empty vector alone. (B) aTRA disrupts the interaction between Daxx and PML-RAR $\alpha$ . The effect of aTRA on Daxx-PML-RAR interaction was determined after a 24-h incubation of the culture in the presence of the indicated concentrations of respective ligands. Columns: 1, solvent only; 2, 1 nM; 3, 10 nM; 4, 100 nM; and 5, 1,000 nM. T3, 3,3',5-triiodo-L-thyronine. (C) Subcellular fractionation of Daxx. HeLa cells were fractionated into cytosolic, membrane, and nuclear fractions, and an equal amount of protein was analyzed by Western blotting for Daxx (left panel). The distribution of the cytoplasmic protein  $\beta$ -tubulin and the nuclear protein hPc2 in each fraction was also determined by immunoblotting to validate the fractionation. Two independent preparations of HeLa nuclear extracts are shown. The right panel is a Coomassie blue-stained gel that shows the relative amount of proteins in each fraction used in the Western blot. (D) Co-IP of Daxx with PML. NB4-cell extracts were immunoprecipitated with affinity-purified anti-Daxx and anti-PML antibodies, and the presence of Daxx in the immunoprecipitates was determined by immunoblotting with anti-Daxx antibodies. The antibodies used for the IP and the Western blot (W.B.) are indicated.

receptor. Since Daxx interacts with both PML and Fas, it is important to determine whether Daxx is a nuclear or cytoplasmic protein. We analyzed the subcellular distribution of Daxx by using biochemical fractionation followed by Daxx immunoblotting. In this assay, Daxx cofractionates primarily with nuclear fraction, with a minority also present in the cytosolic and membrane fractions (Fig. 1C). Control antibodies against the cytoplasmic protein  $\beta$ -tubulin and the nuclear protein polycomb hPc2 (55) show no cross-contamination between the cytoplasmic and nuclear fractions. All of these proteins were detected in the membrane fraction, presumably because this fraction also contains insoluble organelles involved in protein synthesis and transportation. These results demonstrate that Daxx resides mainly in the cell nucleus, suggesting that Daxx may interact with PML in the nucleus.

To confirm that the interaction between Daxx and PML also occurs in mammalian cells, we performed Co-IP assays from HeLa and NB4 cell extracts (Fig. 1D). Both anti-Daxx and anti-PML antibodies, but not preimmune serum, efficiently coimmunoprecipitate endogenous Daxx. These data suggest that Daxx may form a stable complex with PML in vivo. In the immunoprecipitates of Daxx and PML antibodies, we also detected weak signals of the 90-kDa PML and two SUMO-1-conjugated forms of PML (data not shown), confirming the presence of PML in the IP. We also attempted to demonstrate an interaction between Daxx and PML in vitro in GST pull-down and far-Western assays, but all experiments failed to show a convincing interaction. We reasoned that this might be due to the fact that PML is extensively modified by SUMO-1 in

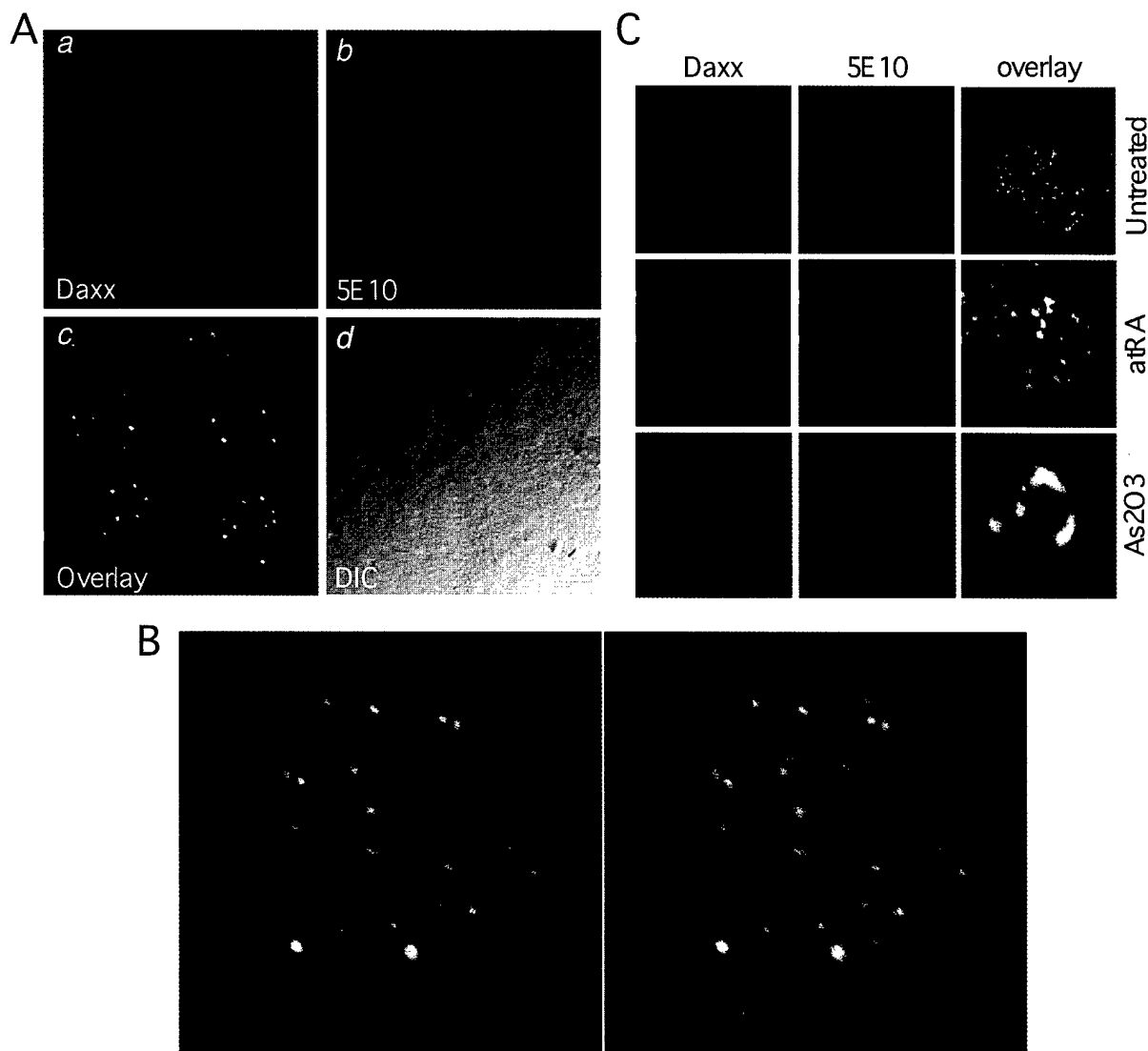


FIG. 2. Daxx colocalizes with PML at the PODs. (A) Confocal immunofluorescence analysis of endogenous Daxx and PML. HEp2 cells were fixed and immunostained with affinity-purified rabbit anti-Daxx polyclonal antibodies and mouse anti-PML 5E10 monoclonal antibodies as described in Materials and Methods. The sample was analyzed by use of a confocal microscope. Panels a and b show the signals of Daxx (green) and PML (red) on a single confocal section. Panel c shows colocalization (yellow signals) of Daxx and PML in the merged image. Panel d is a differential interference contrast image showing the surfaces of the cells and nuclei. Bar, 10  $\mu$ m. (B) Three-dimensional presentation of the colocalization between Daxx and PML. Total of 32 consecutive z-sections at increments of 0.08  $\mu$ m were reconstructed into a three-dimensional image by using the Leica confocal software. The right and left projected images were rotated 4.5° at opposite directions along the x (horizontal) axis. Yellow represents the colocalization between Daxx (green) and PML (red). (C) Colocalization of Daxx and PML in APL cells. NB4 cells were plated on cover glasses coated with poly-L-lysine. The control (untreated), atRA-treated (1  $\mu$ M for 72 h), and As<sub>2</sub>O<sub>3</sub>-treated (1  $\mu$ M for 72 h) cells were fixed and immunostained with anti-Daxx polyclonal and anti-PML monoclonal antibodies. Colocalization of Daxx and PML was revealed by confocal laser microscopy (except for the untreated cells).

vivo (44, 45, 58) or that an additional factor may bridge the interaction between PML and Daxx.

**Daxx colocalizes with PML in the PODs.** We then wished to determine if Daxx colocalizes with PML in the PODs in order to provide further evidence for a physiological interaction between Daxx and PML. Confocal immunofluorescence microscopy using affinity-purified anti-Daxx antibodies reveals discrete nuclear structures in interphase HEp2 cells, in addition to an evenly distributed nucleoplasmic staining (Fig. 2Aa). Double immunostaining, together with use of anti-PML antibodies, demonstrates that the Daxx foci colocalize perfectly with the PODs in cell nuclei (Fig. 2Aa to d). Such colocalization occurs in many different cell types, including HeLa, HEK293, and A549 cells and normal human fibroblasts, sug-

gesting that colocalization between Daxx and PML may be a common phenomenon in different cell types. The colocalization has been confirmed by using antibodies against different POD antigens, including SP100 and SUMO-1, as well as under conditions that modify the POD structure, such as with interferon, As<sub>2</sub>O<sub>3</sub> treatments, and viral infections (unpublished data). A three-dimensional topographic analysis of the colocalization between Daxx and PML demonstrates an extensive colocalization between Daxx and PML in the PODs (Fig. 2B).

**Colocalization of Daxx and PML in NB4 APL cells.** We next analyzed the distribution of Daxx in the NB4 APL cells (Fig. 2C), in which the PODs are disrupted into "microparticulate" structures. Similar to PML, Daxx is also disrupted in the NB4 cells, in which it remains colocalized with PML. The presence

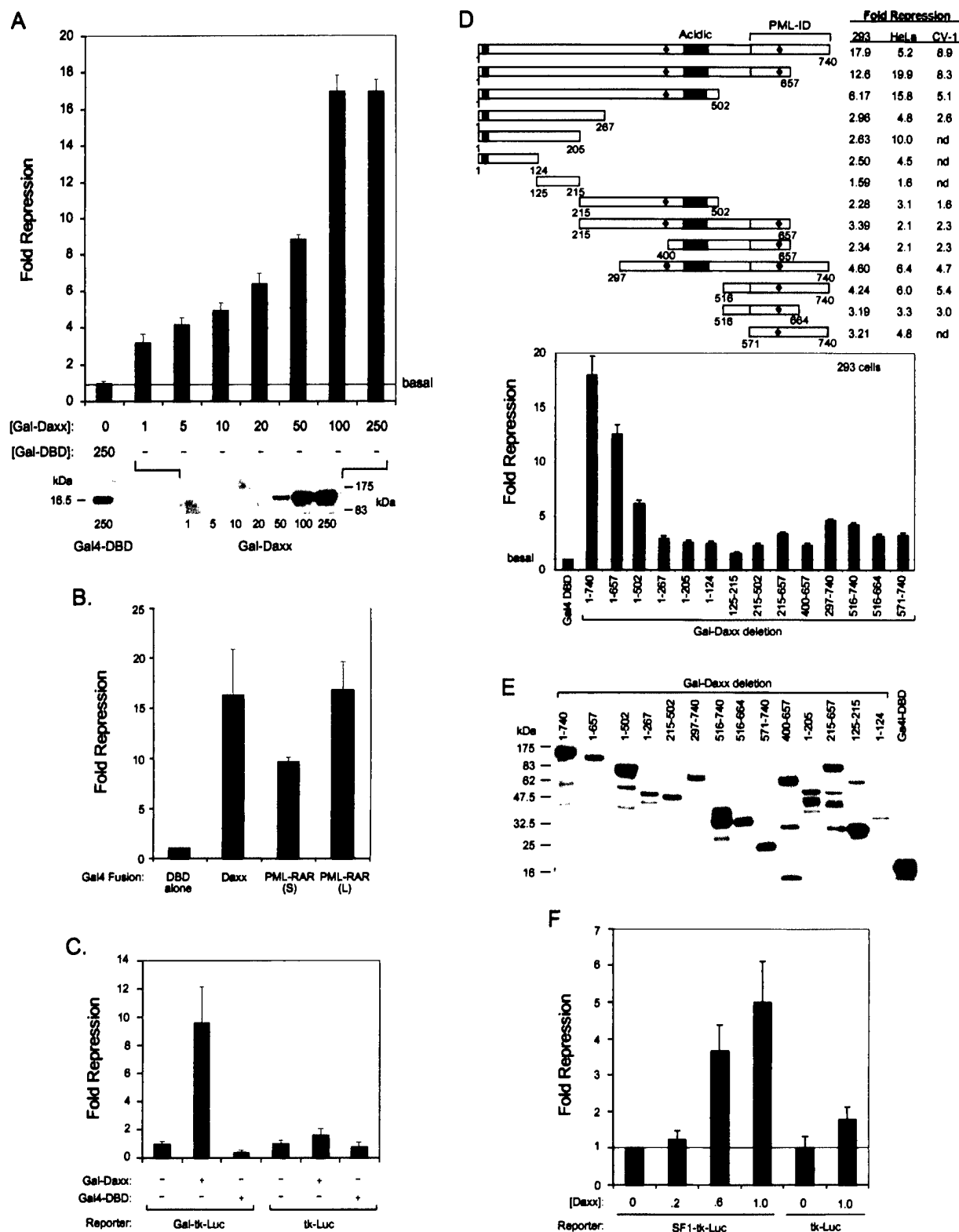


FIG. 3. Modulation of promoter activity by Daxx. (A) Transcriptional repression by Gal-Daxx. Recruitment of Daxx to a promoter via Gal4-DBD results in inhibition of basal transcription in a dose-dependent manner. Transient transfection was conducted in HEK293 cells with increasing concentrations (nanograms) of Gal-Daxx as indicated. The relative fold repression of the basal promoter activity in the presence of Gal-Daxx was compared to that of Gal4-DBD alone. The bottom panels show immunoblots with anti-Gal4-DBD antibodies of the transfected fusion protein at indicated concentrations of expression vector. (B) Daxx represses basal transcription as strongly as PML-RAR $\alpha$ . HEK293 cells were transfected with equal amounts (250 ng) of each expression vector, and the relative repression was determined as described in Materials and Methods. The results show that Gal-Daxx represses basal transcription as strongly as Gal-PML-RAR $\alpha$ . (C) Requirement of binding sites for transcriptional repression by Gal-Daxx. HEK293 cells were transfected with 250 ng of Gal-Daxx or Gal4-DBD alone, and the effects on the promoter activities of Gal-tk-luciferase (luc) and tk-luc reporters were determined. The Gal-tk-luc reporter contains four copies of Gal4-binding sites in front of the minimal tk promoter,

of PML-RAR $\alpha$  fusion protein in the microparticulate structures (17) supports the observed interaction between Daxx and PML-RAR $\alpha$  (Fig. 1). Upon atRA treatment, PML-RAR $\alpha$  is degraded in NB4 cells (47), and these microparticulate structures reorganize into normal size of the PODs (17, 65), where Daxx and PML remain colocalized. The colocalization between Daxx and PML is more evident in NB4 cells treated with As<sub>2</sub>O<sub>3</sub>, in which larger and fewer PODs are observed. These results suggest that Daxx and PML colocalize in APL NB4 cells, and such colocalization persists after reorganization of the PODs induced by atRA or As<sub>2</sub>O<sub>3</sub>.

**Daxx represses basal transcription.** Several POD-associated proteins, including PML, are implicated in transcriptional regulation (for reviews see references 34 and 39). Since Daxx interacts with PML and localizes at the PODs, we decided to test whether Daxx might regulate transcription. Transfection of the Gal4-DBD full-length Daxx fusion protein (Gal-Daxx) in HEK293 cells strongly inhibits basal transcription of the Gal4-tk-luciferase reporter in a dose-dependent manner (Fig. 3A, top). Western blotting using anti-Gal4 DBD antibodies confirms increased expression of Gal-Daxx in transfected cells in the presence of higher concentrations of DNA (Fig. 3A, bottom). Comparison of Daxx-mediated transcriptional repression with that of PML-RAR $\alpha$  fusion proteins indicates that Daxx represses as strongly as the PML-RAR $\alpha$  oncoprotein (Fig. 3B). Moreover, repression by Gal-Daxx requires Gal4-binding sites (Fig. 3C) and occurs in multiple cell types (Fig. 3D), demonstrating the specificity of the observed Daxx-mediated transcriptional repression.

We attempted to determine the sequences in Daxx that are responsible for the repression activity by standard deletion analysis (Fig. 3D). Progressive deletion from the C terminus to residue 124 gradually reduces the repression activity of Daxx in a cell-type-dependent manner. Deletions of the N terminus and several other mutants also show a significant decrease in repression. Equal expression of these Gal-Daxx deletion proteins in transfected cells is confirmed by Western blotting using the anti-Gal4 DBD antibodies (Fig. 3E). These data suggest that multiple regions of Daxx may be important for transcriptional repression in a cell-type-dependent manner.

Daxx was previously isolated in a yeast one-hybrid screen using a reporter containing a SF-1-like element (32). We decided to investigate whether Daxx can repress transcription from a promoter containing the SF-1-like element in a transient-transfection assay (Fig. 3F). As expected, overexpression of wild-type Daxx represses basal transcription from the SF1-tk-luciferase reporter that contains four copies of the SF1-like element, while it has little effect on the tk-luciferase reporter lacking the SF-1 sites. These data indicate that Daxx may repress the basal transcription of natural promoters containing SF1-like elements, a result consistent with the strong repressor activity observed with the Gal-Daxx fusion protein.

**Daxx interacts with HDACs.** Histone deacetylation has been demonstrated to play a central role in transcriptional repression by inducing chromatin assembly and condensation (49, 66). To determine whether histone deacetylation is required

for Daxx-mediated transcriptional repression, we analyzed the interaction between Daxx and the three available human histone deacetylases (HDACs) (67). The three human HDACs are highly conserved in structure and function. All of them repress basal transcription in the Gal4-DBD fusion assay, and all display histone deacetylase activity (67). Far-Western analyses demonstrate interactions between Daxx and all three GST-HDAC fusion proteins, but not GST alone, while PML and SP100 show no interaction with any of these GST-HDACs under the assay conditions (Fig. 4A and B and data not shown). A positive control shows that PML interacts efficiently with GST-PML under identical conditions (Fig. 4C). Furthermore, a Daxx mutant (amino acids 400 to 657) that possesses weak repression activity also does not interact with HDAC1 (Fig. 4C). These data support a role for HDAC interaction in Daxx-mediated transcriptional repression. The interaction between Daxx and HDAC1 is further confirmed in a GST pull-down assay (Fig. 4D), in which GST-HDAC1, but not GST alone, precipitates about 20% of input <sup>35</sup>S-labeled Daxx. Moreover, an interaction between Daxx and HDAC1 *in vivo* is also observed by Co-IP of HeLa nuclear extracts (Fig. 4E), in which HDAC1 coimmunoprecipitates with Daxx antibodies but not with the preimmune serum. Together, these experiments provide strong evidence that Daxx and HDACs interact *in vitro* and *in vivo*.

**HDAC inhibitor reverses Daxx-mediated repression.** The physical interaction observed between Daxx and HDAC suggests that Daxx may recruit a HDAC corepressor complex to repress basal transcription via histone deacetylation and chromatin condensation. To provide more evidence for this possibility, we assayed the effect of a histone deacetylase inhibitor, trichostatin A (TSA), on the repressor activity of Gal-Daxx in a transient-transfection assay (Fig. 4F). As expected, TSA reverses transcriptional repression by Gal-Daxx in a dose-dependent manner, while it has little effect on Gal4-DBD alone under identical conditions. These data indicate that histone deacetylation is involved in transcriptional repression by Daxx.

**Inhibition of Daxx-mediated transcriptional repression by PML.** Since Daxx was identified as a PML-interacting protein and subsequently demonstrated to possess strong transcriptional repression activity, we decided to investigate the role of PML in the regulation of transcriptional repression by Daxx. To do this, Gal-Daxx was cotransfected with increasing amounts of full-length PML into HEK293 cells and the activity of the luciferase reporter was measured (Fig. 5A). As observed above, Gal-Daxx represses reporter expression strongly when compared to the Gal4-DBD alone (Fig. 5A, compare lanes 1 and 6). Interestingly, coexpression of increasing amounts of PML inhibits this repression in a dose-dependent manner, abolishing nearly all of the repressor function of Gal-Daxx (lanes 2 to 5). This effect is specific to Gal-Daxx, for cotransfection of PML with the Gal4-DBD alone has little effect on reporter activity (lanes 7 and 8). These data suggest that PML may inhibit Daxx-mediated transcriptional repression.

Similar experiments were then performed to determine if PML-RAR $\alpha$  might also regulate the function of Gal-Daxx.

while the tk-luc lacks the binding sites. (D) Mapping of the Daxx sequences required for repression. Schematic presentation of Gal-Daxx deletion mutants and their effects on promoter activity in HEK293, HeLa, and CV-1 cells are summarized. The two acidic regions are indicated by black bars, and the two potential nuclear localization signals are marked with diamonds. The bottom graph shows a column presentation of the repression activity of various Gal-Daxx deletion mutants in HEK293 cells. (E) Expression of Gal-Daxx mutants in transfected cells. The transfected lysates were analyzed by immunoblotting by using mouse anti-Gal4-DBD monoclonal antibodies. The top band in each lane represents the expected molecular weights of the Gal-Daxx mutants, except for Gal-Daxx (125-215), where the lower band is the expected product. The deviation in protein expression level was compensated by normalization of luciferase activity with the coexpressed  $\beta$ -Gal activity. (F) Inhibition of basal transcription from a natural promoter by Daxx. Wild-type Daxx was transfected into HEK293 cells together with either the SF1-tk-luciferase or tk-luciferase reporter. The fold repression of the luciferase activity at increasing concentrations (micrograms) of Daxx is presented.

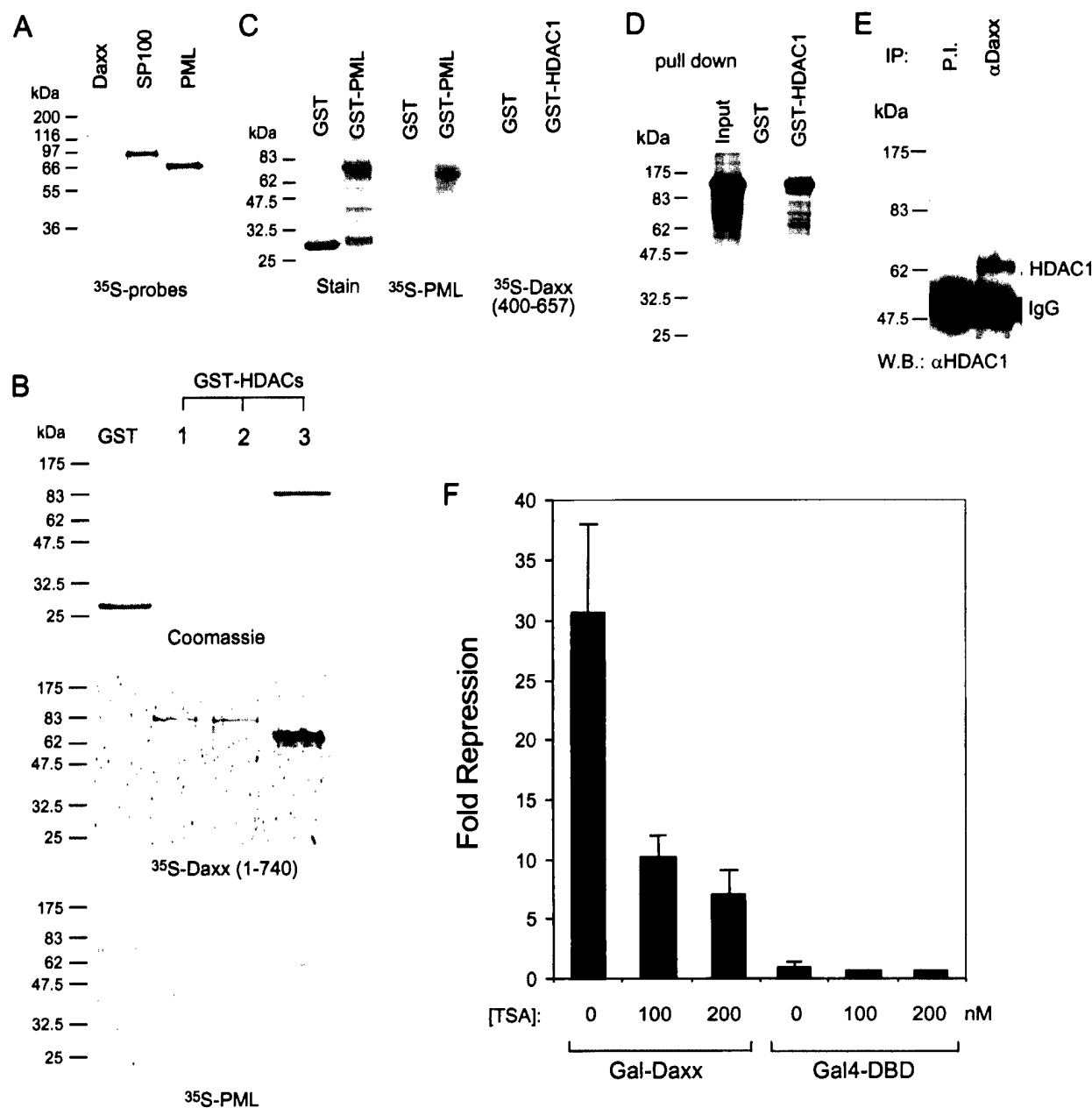
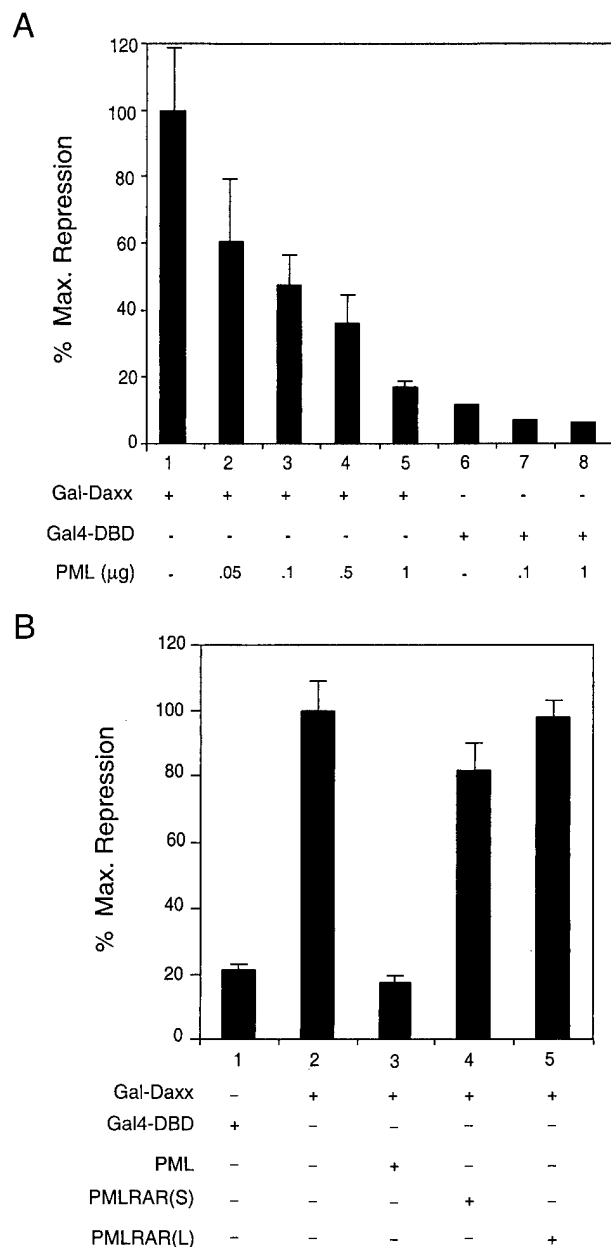


FIG. 4. Interaction of Daxx with HDACs. (A)  $^{35}\text{S}$ -labeled protein probes used in the far-Western assays. In vitro-translated [ $^{35}\text{S}$ ]methionine-labeled Daxx, PML, and SP100 were analyzed by SDS-PAGE and detected by autoradiography. (B) Far-Western analysis of interaction between Daxx and HDACs. The top panel shows the Coomassie blue-stained proteins used in the far-Western assay. The middle panel shows the far-Western blot of GST-HDACs with the  $^{35}\text{S}$ -labeled Daxx probe. The bottom panel shows the far-Western blot with the  $^{35}\text{S}$ -labeled PML probe. (C) PML interacts with GST-PML in the far-Western assay was conducted under conditions identical to those for panel B. A far-Western blot showing that the Daxx mutant (400-657) fails to interact with GST-HDAC1 fusion protein. (D) GST pull-down assay showing interaction between GST-HDAC1 and Daxx. The input  $^{35}\text{S}$ -labeled Daxx contains one-third of the lysate used in the pull-down reaction, which was conducted as described in the Materials and Methods. (E) Co-IP of HDAC1 and Daxx. HeLa nuclear extracts were incubated with affinity-purified anti-Daxx antibody or an equal concentration of the preimmune serum. The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot by using anti-HDAC1 polyclonal antibodies. (F) TSA reverses transcriptional repression by Gal-Daxx. HEK293 cells were transfected with 250 ng of Gal4-DBD or Gal-Daxx mammalian expression vector together with a Gal4-dependent luciferase reporter. The fold repression by Gal-Daxx at different concentrations of TSA was determined relative to that for the Gal4-DBD alone.

When either the short or long forms of PML-RAR $\alpha$  were cotransfected with Gal-Daxx, the repression activity of Gal-Daxx was unchanged (Fig. 5B). Thus, despite the observation that both PML and PML-RAR $\alpha$  interact with Daxx, only PML can inhibit the ability of Daxx to repress transcription, suggesting a differential role of PML and its oncogenic fusion protein in regulation of Daxx function.

**PML recruits Daxx to the POD.** To elucidate the mechanism by which PML blocks Daxx-mediated transcriptional repression, immunofluorescence microscopy was used to investigate the subcellular localization of Gal-Daxx upon coexpression of PML. In these experiments, HEK293 cells were transiently transfected with Gal-Daxx in the absence or presence of HA-PML and subsequently stained with the mouse anti-Gal4-DBD and



**FIG. 5. Inhibition of Daxx-mediated transcriptional repression by PML.** (A) PML inhibits Daxx-mediated transcriptional repression. HEK293 cells were transiently transfected with 100 ng of the Gal4-DBD or Gal-Daxx mammalian expression vectors in the absence or presence of the indicated amounts of PML expression vector together with a Gal4-dependent luciferase reporter. Data are presented as the percentage of maximum repression, where Gal-Daxx activity is represented as 100% repression. (B) PML-RAR $\alpha$  has no effect on Daxx-mediated transcriptional repression. HEK293 cells were transiently transfected with 100 ng of the Gal4-DBD or Gal-Daxx mammalian expression vectors in the absence or presence of expression vectors for PML, PML-RAR $\alpha$  (short form), or PML-RAR $\alpha$  (long form), together with a Gal4-dependent luciferase reporter. Data are presented as the percentage of maximum repression, where Gal-Daxx activity is represented as 100% repression.

rabbit anti-HA antibodies (Fig. 6A). When Gal-Daxx was overexpressed alone in HEK293 cells, a fairly diffuse, evenly distributed staining pattern is observed in the nucleus (Fig. 6A, panels a and b). Cotransfection of PML drastically alters the

distribution of Gal-Daxx, for nearly all of the Gal-Daxx protein is recruited to the PODs, even at very high levels of Gal-Daxx expression (Fig. 6A, panels c to f). Examination of the localization of these enlarged PODs indicates that they occupy the loose chromatin regions (Fig. 6Ae and f), similar to the localization of PODs in the absence of PML overexpression. On the contrary, cotransfection of PML does not recruit a Daxx mutant (Gal-Daxx 1-502) lacking the PML-interacting domain to the PODs (Fig. 6A, panels g to j), suggesting the specificity of the assay. The abilities of PML to reverse Daxx-mediated repression and to recruit Daxx to the PODs support the hypothesis that PML may inhibit Daxx repressor function by sequestration of Daxx to the PODs.

**PML recruits endogenous Daxx to the PODs.** To address whether recruitment of Daxx to the PODs also occurs at the endogenous levels of Daxx, HA-PML was transfected into HEK293 cells alone, and the localization of endogenous Daxx was analyzed by immunofluorescence staining by using anti-Daxx antibodies (Fig. 6B). Double immunostaining with anti-PML antibodies reveals that overexpression of PML leads to accumulation of endogenous Daxx to the PODs, resulting in reduced nucleoplasmic staining (Fig. 6B, panels a to c). Recruitment of endogenous Daxx to the PODs is confirmed with anti-HA antibodies that detect only the transfected HA-PML (Fig. 6B, panels d to f). These data indicate that PML is able to recruit endogenous nucleoplasmic Daxx to the PODs.

**PML does not recruit HDAC1 to the PODs.** So far we have shown that Daxx interacts with HDACs (Fig. 4) and that PML recruits Daxx to the PODs (Fig. 6). Accordingly, we wished to determine the localization of HDAC and other corepressors, such as SMRT, after PML overexpression. We find that overexpression of PML does not alter the distribution of HDAC1 or SMRT (Fig. 6B, panels g to i), suggesting that PML may segregate Daxx away from the corepressor complex. These observations are consistent with a speculative mechanism by which PML may inhibit transcriptional repression of Daxx via sequestration of Daxx to the PODs.

**SUMO-1 modification of PML is required for recruitment of Daxx to the PODs.** To determine if SUMO-1 modification of PML may play a role in Daxx interaction, we generated a PML mutant with all three SUMO-1 modification lysine residues replaced with arginines by site-directed mutagenesis, based on a prior study that mapped the modification sites (31). Upon mutation of the three lysine residues of PML, we no longer observe SUMO-1-conjugated forms of PML, even after treatment of the transfected cells with As<sub>2</sub>O<sub>3</sub> and coexpression with SUMO-1 (Fig. 7A). This PML  $\Delta$ SUMO mutant behaves similarly to the wild-type protein in localizing to the PODs and in enlarging the POD structure (Fig. 7B, panels a, d, and g). Interestingly, while this SUMO-1-deficient mutant is capable of localizing to PODs (panels b and c and panels d and e), overaccumulation of the mutant protein in the PODs fails to recruit nucleoplasmic Daxx (Fig. 7B, panels g to i). In contrast, many of the enlarged PODs show reduced staining of Daxx (Fig. 7B, panels g to i), suggesting that accumulation of the unmodified form of PML in the PODs may lead to the disappearance of Daxx in PODs. These data suggest SUMO-1 modification as being the underlying mechanism for the observed interaction and colocalization between Daxx and PML in vivo.

**SUMO-1 modification of PML is required for efficient inhibition of Daxx-mediated repression.** If our hypothesis that recruitment of Daxx to the POD is inhibitory to its transcriptional repression activity, one would predict that the PML  $\Delta$ SUMO mutant that fails to recruit Daxx to the PODs will be defective in reversing transcriptional repression by Daxx. As expected, we found that the PML  $\Delta$ SUMO mutant is less

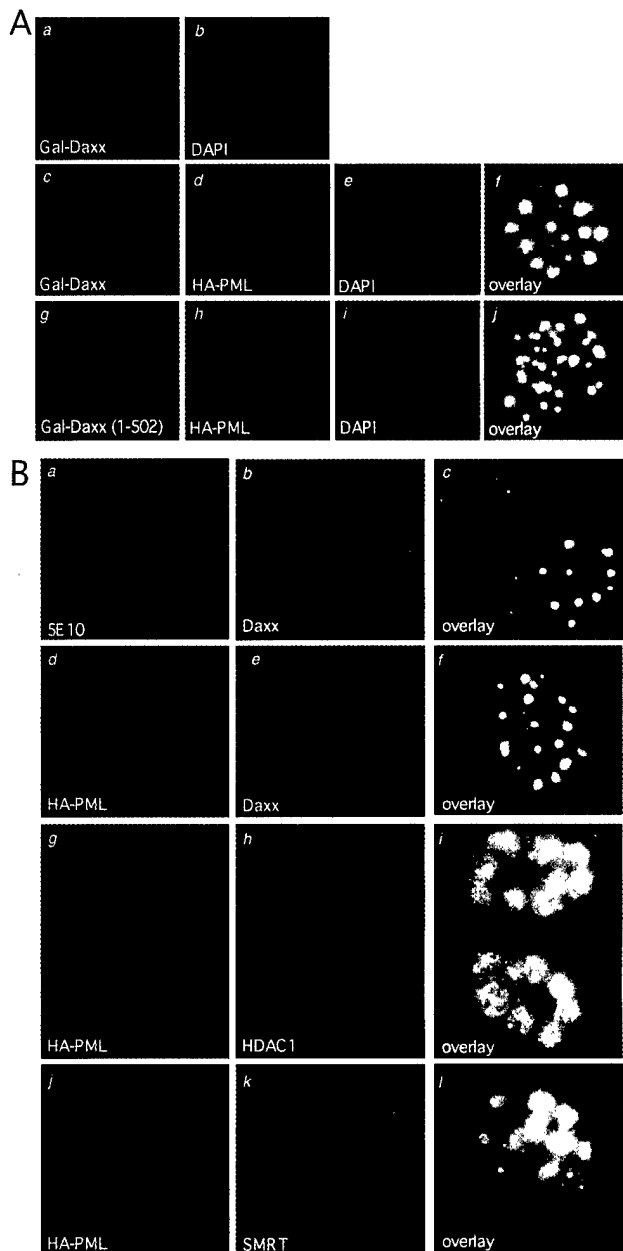


FIG. 6. Recruitment of Daxx to POD domains by overexpression of PML. (A) Overexpression of PML recruits transfected Gal-Daxx into the PODs. Gal-Daxx or Gal-Daxx (1-502) were transiently transfected into Hep2 cells in the absence or presence of HA-tagged PML and subsequently stained with the mouse anti-Gal4-DBD and rabbit anti-HA antibodies. Primary antibodies were detected with rhodamine-conjugated anti-mouse immunoglobulin G and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G secondary antibodies and analyzed by immunofluorescence microscopy. Panels a and b show diffuse nuclear staining of Gal-Daxx in the absence of PML. Panels c to f show Gal-Daxx and HA-PML colocalization at the PODs. Panels g to j show that HA-PML cannot recruit a Gal-Daxx (1-502) mutant lacking the PML-interacting domain to the PODs. (B) Recruitment of endogenous Daxx but not HDAC1 and SMRT to the PODs. HA-PML was transfected into Hep2 cells, and the localization of endogenous Daxx, HDAC1, and SMRT was analyzed by immunofluorescence microscopy. Panels a to c show colocalization of transfected and endogenous PML with endogenous Daxx by using anti-PML monoclonal SE10 and anti-Daxx rabbit polyclonal antibodies. Panels d to f show HA-PML and Daxx colocalization by using the anti-HA monoclonal and anti-Daxx polyclonal antibodies. Panels g to i demonstrate that HA-PML does not recruit HDAC1 or SMRT to the PODs by using anti-HA, anti-HDAC1, or anti-SMRT antibodies. Yellow signals in the overlay images indicate colocalization.

effective in reversing transcriptional repression by Daxx in transient transfection (Fig. 7C). Furthermore, we found that the wild-type PML is incapable of reversing transcriptional repression by Gal-HDAC1 and Gal-SMRTe (Fig. 7C). These data correlate with immunofluorescence studies demonstrating PML recruitment of Daxx, but not HDAC or SMRT, to the POD, where it presumably is unable to repress transcription.

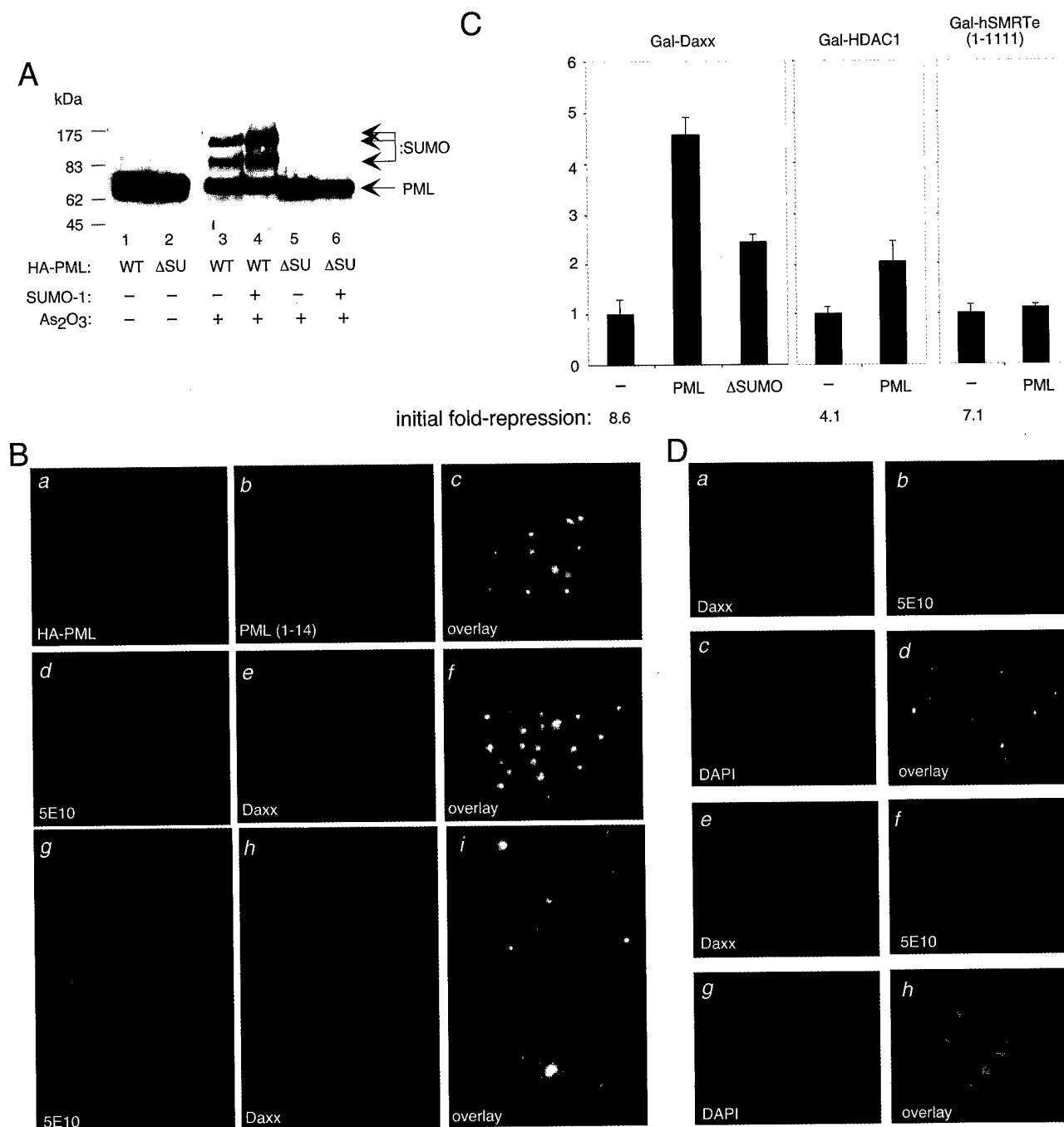
**Daxx is associated with condensed chromatin in the absence of PML.** To provide further evidence that the demonstrated functional interactions between Daxx and PML may be physiologically relevant, we screened several cell lines to find a cell type that may display abnormal localization of Daxx and/or PML. We identified the embryonic carcinoma NT2 cell line; upon staining with the anti-PML antibody, it is evident that only a subset of these cells express PML and thus contain PODs (Fig. 7D). In these cells, PML and Daxx colocalize in the PODs (panels a to d). However, in cells lacking detectable PODs, Daxx forms aggregates around the condensed chromatin (Fig. 7D, panels e to h). Therefore, the localization and thus the function of Daxx may depend on the level of PML in the cell. At low PML levels, Daxx is concentrated at condensed chromatin, where it may repress transcription. When PML levels are higher, it is able to recruit Daxx away from condensed chromatin to the PODs, where Daxx no longer represses basal transcription.

## DISCUSSION

In the present study, we have identified Daxx as a PML-interacting protein and characterized the functional interaction between Daxx and PML. We find a majority of Daxx in the nucleus of HeLa and Hep2 cells where it colocalizes with PML in the PODs. In the NB4 APL cell line, Daxx is distributed in the microparticulate structures that contain the PML-RAR $\alpha$  oncoprotein (17). The repressor function of Daxx is observed upon tethering it to a reporter gene via a heterologous DNA binding domain, as well as from a reporter containing a natural SF1-like promoter element. The mechanism by which Daxx represses basal transcription is found as involving histone deacetylation, for Daxx interacts with HDACs in vitro and in vivo and the histone deacetylase inhibitor, TSA, blocks the repressor activity. Coexpression of PML reverses the transcriptional repression by Daxx, which, in turn, correlates with the recruitment of Daxx to the PODs and efficient inhibition of Daxx-mediated repression. The physiological role of Daxx in transcriptional repression is further supported by the observation that Daxx associates with condensed chromatin in cells that lack PML. Together, these data establish novel roles for Daxx, as a transcriptional repressor, and for PML, as a protein that can potentially regulate the repressor function of Daxx.

Consistent with our findings, Daxx has recently been identified as an inhibitor of transcriptional activation by Pax3, a member of the homeodomain family of transcription factors (28). Thus, Daxx not only is able to repress basal transcription, as suggested from our data, but can also inhibit transcriptional activation via interactions with DNA-binding transcription factors. While the exact mechanism of the inhibition of Pax3 transactivation by Daxx is unclear, our data elucidate the mechanism of Daxx-mediated repression of basal transcription as involving histone deacetylation. We observe Daxx localization to condensed chromatin in NT2 cells that lack detectable PML. Condensed chromatin is considered as a site of transcriptional repression that also includes transcriptionally silent centromeric heterochromatin. Other POD-associated pro-





**FIG. 7. SUMO-1 modification of PML is required for sequestration of Daxx to the POD and inhibition of Daxx-mediated transcriptional repression.** (A) The PML  $\Delta$ SUMO ( $\Delta$ SU) mutant lacks SUMO-1 modification. This mutant was created by replacing all three lysines at residues 65, 160, and 490 with arginines. The wild-type (WT) PML and the  $\Delta$ SUMO mutant were transfected into HEK293 cells alone or in combination with a SUMO-1 expression vector. Cells were treated with 1  $\mu$ M arsenic trioxide ( $As_2O_3$ ) for 6 h where indicated. The total cell lysates were analyzed by Western blotting by using anti-HA monoclonal antibodies. The upper bands in the wild-type proteins represent SUMO-1 conjugated forms of PML. (B) The PML  $\Delta$ SUMO mutant localizes to the PODs but fails to recruit Daxx. The HA-PML  $\Delta$ SUMO mutant was transfected into HEK293 cells and analyzed by immunofluorescence microscopy to detect localization of the transfected mutant protein and the distribution of endogenous Daxx. Panels a to c show localization of the transfected HA-PML  $\Delta$ SUMO mutant protein (detected by a HA antibody) in the PODs that were revealed by a PML polyclonal antibody (1-14). Panels d to f show that the enlarged PODs in the HA-PML  $\Delta$ SUMO mutant transfected cells do not result in prominent recruitment of Daxx to the PODs. Panels g to i show that many enlarged PODs containing PML- $\Delta$ SUMO mutant have little or no Daxx protein. The 5E10 antibodies also detect PODs in untransfected cells that show smaller structures colocalized with Daxx foci. (C) The PML  $\Delta$ SUMO mutant is deficient in reversing transcriptional repression by Daxx. The transfection was conducted in HEK293 cells, and the initial fold repression mediated by the Gal4-DBD fusion proteins is as indicated at the bottom. The y axis indicates the fold reversal of repression. The wild-type PML does not reverse transcriptional repression mediated by HDAC1 or SMRTe. (D) Association of Daxx with condensed chromatin in cells that lack PODs. The human neuronal NT2 stem cells were analyzed by double immunofluorescence staining with anti-Daxx polyclonal and anti-PML 5E10 monoclonal antibodies. The NT2 cells display heterogeneous staining for PML. In cells that contain normal PML nuclear bodies (panels a to d), Daxx appears normal and shows complete colocalization with PML. In contrast, cells that contain only two or fewer PML nuclear dots show aggregated Daxx surrounding the condensed chromatin areas stained with DAPI (panels e to h).

teins, such as SP100, have been demonstrated to interact with heterochromatin protein 1 (HP1) and also colocalize with centromeric chromatin (10, 54). Consistent with this idea, Daxx has been shown to interact with CENP-C in a yeast two-hybrid assay and partially to colocalize with interphase centromeres (50). Also, Daxx has been shown to interact with DNA methyltransferase 1, which plays a role in gene silencing (43).

Previous studies have implicated the PODs as sites of transcriptional activation. For example, PML has been demonstrated to interact with the transcription coactivator CBP and recruit CBP to the PODs (15, 35). Furthermore, PML can enhance the transactivation functions of both CBP and members of the nuclear receptor superfamily (15). PML also induces genes of the major histocompatibility complex, while PML<sup>-/-</sup> mice display reduced transactivation responses to atRA (64, 69). Finally, the transcriptional activator Sp140 (5, 6) and nascent RNA (35) have been found in at least a subset of PODs. Our findings that Daxx represses basal transcription and PML inhibits such repressor activity suggest a new role for the POD structure in gene regulation. The POD may enhance transcription of target genes not only through recruitment of activators but also through the inactivation of repressors such as Daxx via recruitment by PML. Because other transcriptional repressors, such as PLZF, pRB, and Sp100, have also been found in the PODs, it will be interesting to determine if PML can regulate the repressor activities of these proteins as well.

Our observations that PML-RAR $\alpha$  can interact with Daxx but not inhibit transcriptional repression by Daxx suggest a potential role for Daxx in acute promyelocytic leukemia. Support for this hypothesis is evident in our finding that Daxx, PML-RAR $\alpha$ , and PML colocalize at diffusely distributed microparticulate structures in nucleus of the APL NB4 cells. The PML-RAR $\alpha$  fusion protein disrupts the POD structure in these cells and, through its interaction with Daxx, may direct Daxx to the microparticulate structures, where it is capable of repressing gene expression. PML-RAR $\alpha$  itself is a potent transcriptional repressor, which acts via the recruitment of the corepressors SMRT, N-CoR, and HDAC1 (40). The POD structure is reorganized upon treatment of these cells with atRA or arsenic trioxide, leading to the degradation of the PML-RAR $\alpha$  fusion protein and colocalization of Daxx and PML in the PODs (47). Therefore, Daxx inactivation through localization to the PODs may be critical to the differentiation of normal hematopoietic cells. Since expression of the PML-RAR $\alpha$  fusion protein disrupts the integrity of the PODs, Daxx may act as a constitutive repressor in the APL cells, which along with the repressor function of PML-RAR $\alpha$ , may block expression of specific genes that are critical for cell differentiation and culminate in the subsequent APL pathology.

Daxx was initially identified as a Fas-binding protein that promoted Fas-mediated apoptosis via activation of the JNK kinase cascade pathway (12, 68). Interestingly, PML has also been found to be involved in apoptosis triggered by Fas, tumor necrosis factor alpha, and type I and II interferons, possibly by recruitment of the death effector Bax and cdk inhibitor p21 (37, 51, 64). In contrast, expression of PML-RAR $\alpha$  prevents apoptosis in response to these signals (51). Our findings, together with these reports, suggest that the regulation of Daxx repressor function by PML may also be important in programmed cell death. Consistent with this possibility, several transcriptional repressors are known to play a role in apoptosis. For example, the adenovirus E1B and the cellular Bcl-2 oncoprotein block p53-mediated apoptosis by inhibiting transcriptional repression by p53, suggesting that p53 induces apoptosis via transcriptional repression (52, 56). In the case of Daxx, PML may recruit it to the PODs, where it is inactivated,

thus allowing the expression of certain genes required for apoptosis. Conversely, PML-RAR $\alpha$  might inhibit apoptosis in APL cells through disruption of the PODs, thereby promoting enhanced or constitutive repression of these target genes by Daxx and the PML-RAR $\alpha$  fusion protein itself, which leads to the APL phenotype. Retinoic acid treatment would stimulate degradation of PML-RAR $\alpha$  and restoration of the POD structure (17, 47, 65). This would allow Daxx to be inactivated through sequestration to the PODs and allow apoptosis to proceed and would eventually lead to remission of the APL phenotype. Because PML can shuttle between the nucleus and cytoplasm (59, 60), it is possible that Daxx may be brought along with PML to regulate cytoplasmic events relevant to Fas-mediated apoptosis. However, a recent study reports that the loss of Daxx leads to extensive apoptosis in early mouse development (43), a result seemingly opposite to other findings concerning the function of Daxx in apoptosis (12, 13, 68). Therefore, the precise role of Daxx in apoptosis remains to be further elucidated.

Our data provide strong evidence for the roles of PML and the PODs in regulating the function of Daxx as a transcriptional repressor. Daxx and PML interact *in vivo* and colocalize in the PODs. Overexpression of PML recruits Daxx to the PODs, which correlates with a complete inhibition of transcriptional repression by Daxx. Although the detailed mechanism of this inhibition of Daxx by PML remains to be determined, our data provide several possibilities. First, PML might inactivate Daxx by transporting it to the PODs and separating it from HDAC and putative target genes. In response to certain stimuli such as interferon, PML levels increase in the PODs, which, via competition for Daxx binding or conformational change of Daxx upon PML binding, might result in the dissociation of Daxx from HDAC and recruitment of Daxx, but not HDAC, to the PODs. Confinement of Daxx in the PODs would thus block access to target genes, whose expression level would then increase to at least the basal level in the absence of Daxx repression. Our findings that PML overexpression results in increased Daxx levels in the PODs, while having no effect on HDAC1 distribution or repression by HDAC1, support this possibility. Alternatively, the increased PML levels may dissociate HDAC from Daxx and recruit both Daxx and its putative target genes, but not HDAC, to the PODs. Because Daxx requires HDAC and histone deacetylation for its repressor activity, the target genes may be expressed in the absence of HDAC. The presence of transcriptional activators in the PODs would facilitate transcription of target genes. With either possibility, it is evident that the POD is involved in maintaining the balance of Daxx function, depending on the PML level. At normal, physiological levels of PML, Daxx might repress transcription at areas of condensed chromatin. However, with increased PML expression, more Daxx is recruited to the PODs, thus reducing its overall repression activity. Although the precise mechanism of the inhibition of Daxx repression by the PODs awaits further investigation, our data clearly reveal a novel connection between Daxx and PML in regulating transcriptional repression that may play a critical role in acute promyelocytic leukemia and apoptosis.

#### ACKNOWLEDGMENTS

We are grateful to W. M. Yang for the GST-HDAC constructs and to J. F. Strauss III for the SF1-tk-luc construct. We thank M. Lanotte for the NB4 cell line, N. Stuurman for 5E10 monoclonal antibodies, and A. P. Otte for hPc2 antibodies, as well as R. M. Evans for PML-1 and PML-RAR vectors and K. S. Chang for PML vector and antibodies. We also thank colleagues, including W. F. Greenlee, J. Lawrence, D. Ludlum, A. Ross, G. Stein, J. Stein, C. Sagerström, and D. Schroen,

for reading and comments on the manuscript. We thank M. Bhaumik for technical assistance, J. Nickerson for confocal microscopy and M. Nadler for epifluorescence microscopy.

C.L. is a predoctoral fellow of the Army Breast Cancer Program. J.D.C. is a junior scholar of the American Society of Hematology. This work was made possible by grant PRG-98-085-01-LBC from American Cancer Society.

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#### REFERENCES

- Alland, L., R. Muhle, H. Hou, Jr., J. Potes, L. Chin, N. Schreiber-Agus, and R. A. DePinho. 1997. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* 387:49-55.
- Ascoli, C. A., and G. G. Maul. 1991. Identification of a novel nuclear domain. *J. Cell Biol.* 112:785-795.
- Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1995. Short protocols in molecular biology, 3rd ed. John Wiley & Sons, Inc., New York, N.Y.
- Benedetti, L., A. A. Levin, B. M. Scicchitano, F. Grignani, G. Allenby, D. Diverio, F. Lo Coco, G. Avvisati, M. Ruthardt, S. Adamo, P. G. Pelicci and C. Nervi. 1997. Characterization of the retinoid binding properties of the major fusion products present in acute promyelocytic leukemia cells. *Blood* 90:1175-1185.
- Bloch, D. B., J. D. Chiche, D. Orth, S. M. de la Monte, A. Rosenzweig, and K. D. Bloch. 1999. Structural and functional heterogeneity of nuclear bodies. *Mol. Cell Biol.* 19:4423-4430.
- Bloch, D. B., S. M. de la Monte, P. Guigaouri, A. Filippov, and K. D. Bloch. 1996. Identification and characterization of a leukocyte-specific component of the nuclear body. *J. Biol. Chem.* 271:29198-29204.
- Boddy, M. N., K. Howe, L. D. Etkin, E. Solomon, and P. S. Freemont. 1996. PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. *Oncogene* 13:971-982.
- Borden, K. L., M. N. Boddy, J. Lally, N. J. O'Reilly, S. Martin, K. Howe, E. Solomon, and P. S. Freemont. 1995. The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. *EMBO J.* 14:1532-1541.
- Brown, D., S. Kogan, E. Lagasse, I. Weissman, M. Alcalay, P. G. Pelicci, S. Atwater, and J. M. Bishop. 1997. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. *Proc. Natl. Acad. Sci. USA* 94:2551-2556.
- Brown, K. E., S. S. Guest, S. T. Smale, K. Hahn, M. Merckenschlager, and A. G. Fisher. 1997. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* 91:845-854.
- Carvalho, T., J. S. Seeler, K. Ohman, P. Jordan, U. Pettersson, G. Akusjarvi, M. Carmo-Fonseca, and A. Dejean. 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J. Cell Biol.* 131:45-56.
- Chang, H. Y., H. Nishitoh, X. Yang, H. Ichijo, and D. Baltimore. 1998. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science* 281:1860-1863.
- Chang, H. Y., X. Yang, and D. Baltimore. 1999. Dissecting Fas signaling with an altered-specificity death-domain mutant: requirement of FADD binding for apoptosis but not Jun N-terminal kinase activation. *Proc. Natl. Acad. Sci. USA* 96:1252-1256.
- Doucas, V., A. M. Ishov, A. Romo, H. Juguilon, M. D. Weitzman, R. M. Evans, and G. G. Maul. 1996. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev.* 10:196-207.
- Doucas, V., M. Tini, D. A. Egan, and R. M. Evans. 1999. Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling. *Proc. Natl. Acad. Sci. USA* 96:2627-2632.
- Durfee, T., K. Becherer, P. L. Chen, S. H. Yeh, Y. Yang, A. E. Kilburn, W. H. Lee, and S. J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* 7:555-569.
- Dyck, J. A., G. G. Maul, W. H. Miller, Jr., J. D. Chen, A. Kakizuka, and R. M. Evans. 1994. A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* 76:333-343.
- Everett, R. D., and G. G. Maul. 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J.* 13:5062-5069.
- Grande, M. A., I. van der Kraan, B. van Steensel, W. Schul, H. de The, H. T. van der Voort, L. de Jong, and R. van Driel. 1996. PML-containing nuclear bodies: their spatial distribution in relation to other nuclear components. *J. Cell Biochem.* 63:280-291.
- Grignani, F., S. De Matteis, C. Nervi, L. Tomassoni, V. Gelmetti, M. Cioce, M. Fanelli, M. Ruthardt, F. F. Ferrara, I. Zamir, C. Seiser, M. A. Lazar, S. Minucci, and P. G. Pelicci. 1998. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature* 391:815-818.
- Grisolano, J. L., R. L. Wesselschmidt, P. G. Pelicci, and T. J. Ley. 1997. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood* 89:376-387.
- Guidez, F., S. Ivins, J. Zhu, M. Soderstrom, S. Waxman, and A. Zelent. 1998. Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RARalpha underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. *Blood* 91:2634-2642.
- Guldner, H. H., C. Szosteki, T. Grotzinger, and H. Will. 1992. IFN enhance expression of Sp100, an autoantigen in primary biliary cirrhosis. *J. Immunol.* 149:4067-4073.
- Harlow, E., and D. Lane. 1998. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- He, L. Z., F. Guidez, C. Tribioli, D. Peruzzi, M. Ruthardt, A. Zelent, and P. P. Pandolfi. 1998. Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat. Genet.* 18:126-135.
- He, L. Z., C. Tribioli, R. Rivi, D. Peruzzi, P. G. Pelicci, V. Soares, G. Cattoretti, and P. P. Pandolfi. 1997. Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. *Proc. Natl. Acad. Sci. USA* 94:5302-5307.
- Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 1997. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* 387:43-48.
- Hollenbach, A. D., J. E. Sublett, C. J. McPherson, and G. Grosfeld. 1999. The Pax3-FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx. *EMBO J.* 18:3702-3711.
- Kakizuka, A., W. Miller, Jr., K. Umeson, R. Warrell, Jr., S. R. Frankel, V. V. Murty, E. Dmitrovsky, and R. M. Evans. 1991. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* 66:663-674.
- Kalanitry, S., L. Delva, M. Gaboli, D. Gandini, M. Giorgio, N. Hawe, L. Z. He, D. Peruzzi, R. Rivi, C. Tribioli, Z. G. Wang, H. Zhang, and P. P. Pandolfi. 1997. Gene rearrangements in the molecular pathogenesis of acute promyelocytic leukemia. *J. Cell. Physiol.* 173:288-296.
- Kamitani, T., K. Kito, H. P. Nguyen, H. Wada, T. Fukuda-Kamitani, and E. T. Yeh. 1998. Identification of three major sentrinization sites in PML. *J. Biol. Chem.* 273:26675-26682.
- Kiriakidou, M., D. A. Driscoll, J. M. Lopez-Guisa, and J. F. Strauss III. 1997. Cloning and expression of primate Daxx cDNAs and mapping of the human gene to chromosome 6p21.3 in the MHC region. *DNA Cell Biol.* 16:1289-1298.
- Koken, M. H., F. Puvion-Dutilleul, M. C. Guillemin, A. Viron, G. Linares-Cruz, N. Stuurman, L. de Jong, C. Szosteki, F. Calvo, C. Chomienne, et al. 1994. The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO J.* 13:1073-1083.
- Lamond, A. I., and W. C. Earnshaw. 1998. Structure and function in the nucleus. *Science* 280:547-553.
- LaMorte, V. J., J. A. Dyck, R. L. Ochs, and R. M. Evans. 1998. Localization of nascent RNA and CREB binding protein with the PML-containing nuclear body. *Proc. Natl. Acad. Sci. USA* 95:4991-4996.
- Lanotte, M., V. Martin-Thouvenin, S. Najman, P. Balerini, F. Valensi, and R. Berger. 1991. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood* 77:1080-1086.
- Le, X. F., S. Vallian, Z. M. Mu, M. C. Hung, and K. S. Chang. 1998. Recombinant PML adenovirus suppresses growth and tumorigenicity of human breast cancer cells by inducing G1 cell cycle arrest and apoptosis. *Oncogene* 16:1839-1849.
- Li, H., P. J. Gomes, and J. D. Chen. 1997. RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC1 and TIF2. *Proc. Natl. Acad. Sci. USA* 94:8479-8484.
- Lin, R. J., D. A. Egan, and R. M. Evans. 1999. Molecular genetics of acute promyelocytic leukemia. *Trends Genet.* 15:179-184.
- Lin, R. J., L. Nagy, S. Inoue, W. Shao, W. H. Miller, Jr., and R. M. Evans. 1998. Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 391:811-814.
- Mahajan, R., C. Delphin, T. Guan, L. Gerace, and F. Melchior. 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88:97-107.
- Maul, G. G., A. M. Ishov, and R. D. Everett. 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* 217:67-75.
- Michaelson, J. S., D. Bader, F. Kuo, C. Kozak, and P. Leder. 1999. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev.* 13:1918-1923.
- Mu, Z. M., X. F. Le, S. Vallian, A. B. Glassman, and K. S. Chang. 1997. Stable overexpression of PML alters regulation of cell cycle progression in HeLa cells. *Carcinogenesis* 18:2063-2069.
- Muller, S., M. J. Matunis, and A. Dejean. 1998. Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. *EMBO J.* 17:61-70.

46. Nagy, L., H. Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**:373–380.
47. Nervi, C., F. F. Ferrara, M. Fanelli, M. R. Rippon, B. Tomassini, P. F. Ferrucci, M. Ruthardt, V. Gelmetti, C. Gambacorti-Passerini, D. Diverio, F. Grignani, P. G. Pelicci, and R. Testi. 1998. Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARalpha fusion protein. *Blood* **92**:2244–2251.
48. Okura, T., L. Gong, T. Kamitani, T. Wada, I. Okura, C. F. Wei, H. M. Chang, and E. T. Yeh. 1996. Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. *J. Immunol.* **157**:4277–4281.
49. Pazin, M. J., and J. T. Kadonaga. 1997. What's up and down with histone deacetylation and transcription? *Cell* **89**:325–328.
50. Pluta, A. F., W. C. Earnshaw, and I. G. Goldberg. 1998. Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. *J. Cell Sci.* **111**:2029–2041.
51. Quignon, F., F. De Bels, M. Koken, J. Feunteun, J. C. Ameisen, and H. de The. 1998. PML induces a novel caspase-independent death process. *Nat. Genet.* **20**:259–265.
52. Sabbatini, P., S. K. Chiou, L. Rao, and E. White. 1995. Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. *Mol. Cell. Biol.* **15**:1060–1070.
53. Satijn, D. P., M. J. Gunster, J. van der Vlag, K. M. Hamer, W. Schul, M. J. Alkema, A. J. Saurin, P. S. Freemont, R. van Driel, and A. P. Otte. 1997. RING1 is associated with the polycomb group protein complex and acts as a transcriptional repressor. *Mol. Cell. Biol.* **17**:4105–4113.
54. Seeler, J. S., A. Marchio, D. Sitterlin, C. Transy, and A. Dejean. 1998. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc. Natl. Acad. Sci. USA* **95**:7316–7321.
55. Sewalt, R. G., J. van der Vlag, M. J. Gunster, K. M. Hamer, J. L. den Blaauwen, D. P. Satijn, T. Hendrix, R. van Driel, and A. P. Otte. 1998. Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. *Mol. Cell. Biol.* **18**:3586–3595.
56. Shen, Y., and T. Shenk. 1994. Relief of p53-mediated transcriptional repression by the adenovirus E1B 19-kDa protein or the cellular Bel-2 protein. *Proc. Natl. Acad. Sci. USA* **91**:8940–8944.
57. Shen, Z., P. E. Pardington-Purtymun, J. C. Comeaux, R. K. Moyzis, and D. J. Chen. 1996. UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* **36**:271–279.
58. Sternsdorf, T., K. Jensen, and H. Will. 1997. Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J. Cell Biol.* **139**:1621–1634.
59. Stuurman, N., A. de Graaf, A. Floore, A. Jossen, B. Humbel, L. de Jong, and R. van Driel. 1992. A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J. Cell Sci.* **101**:773–784.
60. Stuurman, N., A. Floore, E. Middelkoop, R. van Driel, and L. de Jong. 1997. PML shuttles between nuclear bodies and the cytoplasm. *Cell Mol. Biol. Lett.* **2**:137–150.
61. Szekely, L., K. Pokrovskaja, W. Q. Jiang, H. de The, N. Ringertz, and G. Klein. 1996. The Epstein-Barr virus-encoded nuclear antigen EBNA-5 accumulates in PML-containing bodies. *J. Virol.* **70**:2562–2568.
62. Szosteck, C., H. H. Guldner, H. J. Netter, and H. Will. 1990. Isolation and characterization of cDNA encoding a human nuclear antigen predominantly recognized by autoantibodies from patients with primary biliary cirrhosis. *J. Immunol.* **145**:4338–4347.
63. Wang, Z. G., L. Delva, M. Gaboli, R. Rivi, M. Giorgio, C. Cordon-Cardo, F. Grosveld, and P. P. Pandolfi. 1998. Role of PML in cell growth and the retinoic acid pathway. *Science* **279**:1547–1551.
64. Wang, Z. G., D. Ruggero, S. Ronchetti, S. Zhong, M. Gaboli, R. Rivi, and P. P. Pandolfi. 1998. Pml is essential for multiple apoptotic pathways. *Nat. Genet.* **20**:266–272.
65. Weis, K., S. Rambaud, C. Lavau, J. Jansen, T. Carvalho, M. Carmo-Fonseca, A. Lamond, and A. Dejean. 1994. Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. *Cell* **76**:345–356.
66. Wolffe, A. P. 1997. Sinful repression. *Nature* **387**:16–17.
67. Yang, W. M., Y. L. Yao, J. M. Sun, J. R. Davie, and E. Seto. 1997. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. *J. Biol. Chem.* **272**:28001–28007.
68. Yang, X., R. Khosravi-Far, H. Y. Chang, and D. Baltimore. 1997. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89**:1067–1076.
69. Zheng, P., Y. Guo, Q. Niu, D. E. Levy, J. A. Dyck, S. Lu, L. A. Sheiman, and Y. Liu. 1998. Proto-oncogene PML controls genes devoted to MHC class I antigen presentation. *Nature* **396**:373–376.